IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Masaya YAMANOUCHI et al.

Application No.: 09/578,693 — Confirmation No.: 9841

Filed: May 26, 2000 — Art Unit: 1641

For: METHOD FOR EXAMINING HUMAN KIDNEY DISEASES BY DETECTING THE FATTY ACID BINDING PROTEIN — Examiner: L. V. Cook

APPEAL BRIEF TRANSMITTAL FORM

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith is an Appeal Brief on behalf of the Appellants in connection with the above-identified application.

☐ The enclosed document is being transmitted via the Certificate of Mailing provisions of 37 C.F.R. § 1.8.

A Notice of Appeal was filed on November 13, 2006.

☐ Applicant claims small entity status in accordance with 37 C.F.R. § 1.27.

The fee has been calculated as shown below:

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If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Dated: June 8, 2007

Respectfully submitted,

By _____ #42,874

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MS APPEAL BRIEF - PATENTS
Docket No.: 0020-4710P
(PATENT)

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APPEAL BRIEF ON BEHALF OF APPELLANTS UNDER 37 C.F.R. §41.37

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APPEAL BRIEF ON BEHALF OF APPELLANTS UNDER 37 C.F.R. § 41.37

MS APPEAL BRIEF-PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

INTRODUCTORY COMMENTS

Acknowledging that the Notice of Appeal was filed on November 13, 2006, and the period having been extended five months to June 13, 2007, Appellants request reconsideration and reversal of the Final Rejection dated June 13, 2006 of the present claims by the Honorable U.S. Board of Appeals and Patent Interferences.

I. REAL PARTY IN INTEREST

As evidenced by the Assignment filed July 26, 2000 and recorded at Reel 11006, Frames 0891-0893 the Real Party in Interest in the present application is the assignee of record, Tanabe Seiyaku Co., Ltd. of Osaka Japan.

II. RELATED APPEALS AND INTERFERENCES

There are no pending Appeals or Interferences related to the present application known to Appellants or Appellants' Legal Representatives.

III. STATUS OF THE CLAIMS

Claims 2, 4, 6, 9, 16-19, 21-24 and 27 are pending and rejected. Claims 1, 3, 5, 7, 8, 10-15, 20, 25 and 26 have been previously cancelled. Claims 2, 4, 6, 9, 16-19, 21-24 and 27 are appealed. The claims do not stand or fall together and are grouped as outlined below.

IV. STATUS OF AMENDMENTS

All amendments presented by Appellants have been entered. Further, no amendments have been filed subsequent to the Final Office Action dated June 13, 2006, therefore the status of amendments is as displayed in the Claims appendix attached hereto.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Two independent claims are presently appealed, claims 16 and 24. Independent claim 16 relates to a method for diagnosis or prognosis of a kidney disease in a human (page 4, lines 14-16 and page 5, lines 14-15). This method commences with the step of preparing a specimen collected from a human. The specimen to which the present method is applied is either kidney tissue or urine (page 8, lines 8-14). Next, liver-type fatty acid binding protein contained in the specimen is detected (page 4, line 22 to page 5, line 1). It is known that different types of fatty acid binding proteins (FABPs) are known. These include liver-type, heart-type and kidney-type fatty acid binding protein. It has been reported that in human kidney tissue, at least two FABPs are expressed, which are liver-type fatty acid binding protein (L-FABP) and heart muscle-type fatty acid binding protein (H-FABP). L-FABP distributes at the proximal tubule, while H-FABP distributes mainly at the distal tubule. See page 6, lines 2-14. The present inventors have discovered that the existence of L-FABP is closely related with the prognosis of kidney disease and thus allow the third step of diagnosing or prognosing kidney disease based on the test result of the detection above. According to this method, the specimen is kidney tissue or urine.

Independent claim 24 relates to a method for diagnosing the progression of kidney disease in a patient suffering therefrom (page 4, lines 14-16, page 5, lines 14-15, and page 21, line 14 to page 22, line 4). The method involves preparing a specimen collected from the patient. This specimen is either kidney tissue or urine (page 8, lines 8-14). Then, liver-type fatty acid binding protein contained in the specimen is detected (page 4, line 22 to page 5, line 1). Next, it

is possible to diagnose the progression of the kidney disease based on the test result of the detection.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

There are three grounds of rejection to be reviewed on appeal, which are:

(1) Whether, under 35 U.S.C. §103(a), claims 2, 4, 6, 16, 17, 18, 22, 23, 24 and 27 are obvious over Gorski et al. (*Clinical Chemistry*, 43(1):193-195, 1997) (herein "Gorski") in view of Maatman et al. (*Biochem. J.* 288:285-290, 1992) (herein "Maatman") and Simon et al. (*J. Biol. Chem.* 272(16):10652-10663, 1997) (herein "Simon").

(2) Whether, under 35 U.S.C. §103(a), claim 9 is obvious over Gorski in view of Maatman and Simon and further in view of Kimura et al. (*J. Biol. Chem.*, 266(9):5963-5972, 1991) (herein "Kimura").

(3) Whether, under 35 U.S.C. § 103(a), claims 19 and 21 are obvious over Gorski in view of Maatman and Simon and further in view of Galaske et al. (*Pflugers Archives Euro. J. Physiol.*, 375(3):269-277, 1978) (herein "Galaske").

VII. ARGUMENT

Appellants respectfully submit that the claims subject to this appeal have been improperly rejected under 35 U.S.C. § 103(a). Appellants will address the three grounds of rejection in turn below. As part of these arguments, the grouping of claims will be discussed at the beginning of each of the three sections. As will be seen, the appealed claims do not stand or fall together.

The Present Invention and its Advantages

Prior to the present invention, there existed no method for diagnosis or prognosis or examination of a patient utilizing a relationship between liver-type fatty acid binding protein (L-FABP) in kidney tissues or urine and kidney diseases. That is, the present inventors have been the first to discover a relation between L-FABP and kidney diseases. Thus, the present invention relates to a method for examining kidney disease, which involves detection of L-FABP contained in the specimen, which is either kidney tissue or urine. The present invention thus provides for the prognosis and diagnosis of various kidney diseases, for instance, diabetic nephropathy, glomerulonephritis, nephrotic syndrome, focal glomerulosclerosis, immune complex nephropathy (IgA nephropathy, membranous nephropathy, etc.), lupus nephritis, drug-induced renal injury, renal insufficiency and kidney graft rejection.

(1) Claims 2, 4, 6, 16, 17, 18, 22, 23, 24 and 27 are non-obvious over the references of Gorski, Maatman and Simon.

Appellants respectfully submit that claims 2, 4, 6, 16, 17, 18, 22, 23, 24 and 27 are non-obvious over the prior art cited by the Examiner. As such, the Board is respectfully requested to reverse the rejection discussed under section (VI)(1) above.

Independent Claim 16

When a rejection is based on 35 U.S.C. §103(a), what is in issue in such a rejection is "the invention as a whole," not just a few features of the claimed invention. Under 35 U.S.C. §103, "[a] patent may not be obtained . . . if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains." The determination under §103 is whether the claimed invention as a whole would have been obvious to a person of ordinary skill in the art at the time the invention was made. See In re O'Farrell, 853 F.2d 894, 902, 7 USPQ2d 1673, 1680 (Fed. Cir. 1988). In determining obviousness, the invention must be considered as a whole and the claims must be considered in their entirety. See Medtronic, Inc. v. Cardiac Pacemakers, Inc., 721 F.2d 1563, 1567, 220 USPQ 97, 101 (Fed. Cir. 1983).

In rejecting claims under 35 U.S.C. §103, it is incumbent on the examiner to establish a factual basis to support the legal conclusion of obviousness. See, In re Fine, 837 F.2d 1071, 1073, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988). In so doing, the examiner is expected to make the factual determinations set forth in Graham v. John Deere Co., 383 U.S. 1, 17, 148 USPQ 459, 467 (1966), and to provide a reason why one of ordinary skill in the pertinent art would have

been led to modify the prior art or to combine prior art references to arrive at the claimed invention. Such reasoning must stem from some teaching, suggestion or implication in the prior art as a whole or knowledge generally available to one having ordinary skill in the art. Uniroyal Inc. v. F-Wiley Corp., 837 F.2d 1044, 1051, 5 USPQ2d 1434, 1438 (Fed. Cir. 1988), cert. denied, 488 U.S. 825 (1988); Ashland Oil, Inc. v. Delta Resins & Refractories, Inc., 776 F.2d 281, 293, 227 USPQ 657, 664 (Fed. Cir. 1985), cert. denied, 475 U.S. 1017 (1986); ACS Hospital Systems, Inc. v. Montefiore Hospital, 732 F.2d 1572, 1577, 221 USPQ 929, 933 (Fed. Cir. 1984). These showings by the examiner are an essential part of complying with the burden of presenting a *prima facie* case of obviousness. Note, In re Oetiker, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). A *prima facie* case of obviousness is not established unless the prior art suggests the desirability of the modification. In re Brouwer, 77 F.3d 422, 37 USPQ2d 1163 (Fed. Cir. 1995). Stated differently, the mere fact that the prior art may be modified in the manner suggested by the examiner does not make the modification obvious unless the prior art suggested the desirability of the modification. In re Fritch, 972 F.2d 1260, 1266, 23 USPQ2d 1780, 1783-84 (Fed. Cir. 1992).

Many relevant cases have been discussed above and all must be given due consideration. Additionally, the recent decision of the Supreme Court in KSR International Co. v. Teleflex Inc., 550 U.S. (2007), 82 USPQ2d 1385 (US 2007), although not decided at the time of the Examiner's Final rejection, and although not aiding in strengthening the position taken by the Examiner, should not be ignored. According to *KSR*, the "obvious to try" rationale can form the basis for an obviousness rejection, but only under certain circumstances. As will be seen, these

circumstances are not currently applicable. For instance, according to the Supreme Court in KSR International:

> When there is a design need or market pressure to solve a problem and there are a finite number of identified predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product was not of innovation but of ordinary skill and common sense. In that instance, the fact that the combination was obvious to try might show that it was obvious under §103(a)." KSR International, 82 USPQ2d at 1397.

In the present instance and rejections as formulated by the Examiner, the issue of a finite number of identified predictable solutions never arouse. There was simply no motivation to utilize liver-type fatty acid binding protein to diagnose or prognose kidney disease. Therefore, an obvious to try rationale cannot form the basis for a §103(a) rejection in the present instance.

The Examiner's rejection relies on the combined references of Gorski, Maatman and Simon. Appellants respectfully submit that these references, whether taken individually or in combination, fail to suggest or disclose the subject matter of claim 16. Appellants will first discuss the references cited by the Examiner, and then discuss the patentability of claim 16.

Gorski discloses a study on the concentrations of heart-type FABP (H-FABP) in plasma of patients with chronic renal failure. Gorski does not discuss detection of liver-type FABP (L-FABP). The Examiner's assertion is that one of ordinary skill in the art would utilize the teachings of Maatman and Simon, relating to L-FABP to modify the reference of Gorski. However, Appellants respectfully submit that there exists no motivation to replace the H-FABP of Gorski with the L-FABP of Maatman or Simon. Applicants remind the Examiner that a *prima facie* case of obviousness is not established unless the prior art suggests the desirability of the modification. In re Brouwer, 77 F.3d 422, 37 USPQ2d 1163 (Fed. Cir. 1995); see also In re

Fritch, 972 F.2d 1260, 1266, 23 USPQ2d 1780, 1783-84 (Fed. Cir. 1992). Nevertheless, as will be discussed in detail below, there is no indication that H-FABP and L-FABP are similar or equivalent.

The secondary reference of Maatman simply discloses that the content of H-FABP and L-FABP mRNAs in the kidney is similar. However, similar transcription levels do not necessarily correlate with similar function, and the amino acid homology between H-FABP an L-FABP is low. Thus, one of ordinary skill in the art would not consider H-FABP and L-FABP to be interchangeable. Further, Appellants respectfully submit that Maatman only provides mere speculation as to the function of L-FABP, and this speculation cannot provide adequate motivation for one of ordinary skill in the art to replace the H-FABP of Gorski with the L-FABP of Maatman.

In order to further discuss the deficiencies of the Examiner's rejection, Appellants will summarize the teachings of Gorski, Maatman and Simon. The Examiner relies on each of those references in each rejection. Please note that although Appellants are addressing these references individually, it is submitted that even the combined disclosure of these references cannot render claim 16 *prima facie* obvious.

Gorski

Gorski discloses that "Plasma FABP concentration is markedly increased in patients with chronic renal failure." The study of Gorski was done by focusing attention on FABP as a marker for myocardial infarction, and the FABP of Gorski is "heart-type FABP (H-FABP)", as will be discussed further below. The Gorski reference is completely silent concerning "liver-type FABP

(L-FABP)". Thus, Gorski fails to suggest or disclose the concept of diagnosis of kidney disease by using FABP much less L-FABP.

In Gorski throughout the entire disclosure, from the introduction (the object of research) to the final conclusion, FABP (H-FABP) is treated as a marker for the diagnosis of myocardial infarction. Gorski was attempting to show that the diagnosis of myocardial infarction in patients with chronic renal failure may be affected by erroneous interpretation of data. Gorski obtained data for patients with normal heart function and chronic renal failure as the preinfarction data. In the conclusion Gorski discusses renal failure merely in relation with the diagnosis of myocardial infarction. (See Gorski, page 194, left column, lines 4-8; page 194, left column, lines 8-30; page 195, left column, lines 12-21; and page 195, left column, lines 25-36). Further, although the Examiner has pointed out that Gorski teaches urine measurement of increased FABP (H-FABP as discussed above) (see page 193, left column lines 20-21 of Gorski), this measurement simply concerns measurement of urine with respect to myocardial infarction.

At this point, Appellants point out that one skilled in the art would understand that the disclosure of Gorski is completely focused on the diagnosis of myocardial infarction. In order to reach to the subject matter of claim 16, it is necessary to change the direction to the diagnosis of kidney disease which is completely lacking from the cited references and further replacing H-FABP with L-FABP. The disclosure of Gorski would not lead one skilled in the art to the present invention.

Maatman

Maatman discloses the existence of L-FABP and H-FABP in human kidneys, and further include speculation that L-FABP may prevent nephrotoxicity by binding with drugs. However,

the "possibility" of preventing nephrotoxicity with L-FABP is not related to the diagnosis of a kidney disease with L-FABP. Specifically, as will be discussed below, the possibility of preventing nephrotoxicity with L-FABP does not suggest the possibility of diagnosis of kidney disease. Thus, Maatman fails to suggest or disclose the diagnosis of kidney disease in a human. Further, Appellants submit that the H-FABP and L-FABP of Maatman are not considered to be similar to, or equivalent to each other.

Simon

The Simon reference is concerned with control of the expression of L-FABP. Further, Simon disclose that a DNA sequence ("heptad repeat") existing in an upstream region of the L-FABP gene has a function of suppressing expression of L-FABP in mouse kidney. However, Simon fails to suggest or disclose the diagnosis of kidney disease in a human.

Briefly, none of Gorski, Maatman, or Simon suggests or discloses the diagnosis of kidney disease in a human using L-FABP. This represents a major deficiency in the Examiner's rejection. Further, Appellants submit that there exist numerous problems in combining Gorski with Maatman and/or Simon, and in replacing the H-FABP of Gorski with L-FABP to reach to the subject matter of claim 16. This is discussed further below:

(i) FABP in Gorski is H-FABP

From the opening phrase of Gorski, it is clear that the FABP disclosed therein is H-FABP:

> Heart and skeletal muscles contain the same type of FABP (referred to as heart-type (H)-FABP [1, 2], but its concentration in the heart is several fold higher than that in the skeletal muscles [3]. The concentration of FABP in the plasma of healthy persons is relatively low (2-6 μ-L^{-1}) [4]. FABP is released from the heart early after the onset of infarction, whereafter its plasma concentration increases

> many fold [3-6]. Increased exertion of FABP in urine also occurs after infarction [5-7]. Several recent studies indicate the usefulness of plasma FABP concentration as an early biochemical marker for myocardial infarction diagnosis [3,5,7]. *See page 193, right column of Gorski.*

As is clear from this opening passage, the FABP focused on by Gorski is an FABP which functions as a marker for myocardial infarction diagnosis and is released from the heart after the onset of infarction. This FABP is an FABP existing in the heart, that is, H-FABP.

Further, it is clear that FABP in Gorski is H-FABP in view of the method used by Gorski to measure FABP. Specifically, on page 194, middle column, lines 13-15, Gorski describes the experimental method as, "Plasma FABP concentration as measured by **sensitive noncompetitive sandwich ELISA** [4] (emphasis added)." The reference [4] cited by Gorski is Wodzig et al, *Ann. Clin. Biochem.*, 34:263-268, 1997 (filed as Exhibit 1 with the Reply under 37 C.F.R. §1.111 dated July 22, 2003).

In Wodzig et al., the method for measuring plasma FABP is disclosed. Specifically, in the summary, pages 1-3, Wodzig et al. discloses:

> To allow a more rapid determination of heart-type fatty acid-binding protein (FABP) concentration in plasma a **direct non-competitive (sandwich-type) ELISA was developed which uses high-affinity monoclonal antibodies to FABP** (emphasis added).

Furthermore, on page 264, left column, lines 27-43, Wodzig et al. adds:

> **For measurement of FABP in serum or plasma, a direct non-competitive ELISA of antigen capture type (sandwich ELISA) was developed, based on the use of monoclonal antibodies (mAb).** Thirteen mAbs, all of subtype IgGI and directed against **purified human heart-type FABP** were raised by the classical hybridoma technology and characterized by surface plasma resonance analysis using a Pharmacia BIA core biosensor, as described in detail elsewhere. The mAbs recognized five distinct (three independent and two overlapping) epitopes on human FABP and **showed no cross-reactivity with human intestinal-type and human liver-type FABP.** Seven of mAbs were selected (emphasis added)

As noted in the above passage, the monoclonal antibodies (mAbs) utilized in the non-competitive sandwich ELISA are directed against human H-FABP. These monoclonal antibodies show no cross-reactivity with human L-FABP. Thus, the direct non-competitive (sandwich-type) ELISA disclosed in Wodzig et al. and utilized by Gorski measures only H-FABP concentration in plasma. In other words, since the monoclonal antibodies do not cross-react with L-FABP, it is not possible to measure L-FABP with this method. In view of Wodzig et al., therefore, the "FABP" referred to by Gorski is clearly H-FABP.

H-FABP and L-FABP are clearly different proteins and differences in H-FABP and L-FABP are supported by Van Nieuwenhovern et al., *Lipids*, Vol. 31 Suppl: S233-S227, 1996 (cited as [2] in Gorski et al.) (filed as Exhibit 2 with Reply under 37 CFR §1.111 dated July 22, 2003) and Veerkamp et al., Prog. Lipid Res., 34(1):17-52, 1995 (filed as Exhibit 3 with Reply under 37 CFR §1.111 dated July 22, 2003). Van Nieuwenhovern et al. lists the various FABPs on page s225, Table 2. Veerkamp et al. lists the various FABPs on page 21, Table 3. In both of these tables, H-FAPB and L-FABP are classified as different proteins. Furthermore, Veerkamp et al. discloses the amino acid sequence alignment between human heart-type FABP and human liver-type FABP on page 23, Figure 2. It is clear from the alignment that the two FABP proteins do not share a high homology.

In summary, it is understood by the skilled artisan that the FABP measured by noncompetitive sandwich ELISA and discussed in Gorski is H-FABP, even though Gorski merely refers to the H-FABP as simply "FABP." Accordingly, it is clear that the FABP (H-FABP) in Gorski is clearly different from the L-FABP the present invention.

(ii) H-FABP and L-FABP are not similar to or equivalent to each other.

The Examiner apparently considers that H-FABP and L-FABP are similar and/or functional equivalent (do not markedly differ). Thus, the Examiner appears to believe that it is easy to replace H-FABP with L-FABP.

However, H-FABP and L-FABP are neither similar nor equivalent (nor functionally equivalent) whether viewed from the standpoint of Maatman or from the standpoint of Gorski. Firstly, Maatman disclose the existence of both of H-FABP and L-FABP in the kidney but also clearly mentions the difference of H-FABP and L-FABP. That is, it is clearly mentioned that H-FABP and L-FABP are different in ligand specificities and cellular distributions. The difference in ligand specificities will be understood from the following description:

> The significance of the occurrence in kidney of two FABP types with different ligand specificities and cellular distributions requires further investigation. Page 289, right column, 2nd paragraph, lines 10-12 of Maatman.

> The liver-type FABP also binds some drugs [2,3], and may in this way prevent nephrotoxicity. The heart-type FABP only binds fatty acids and seems to be involved in lipid metabolism. Page 289, right column, 1st paragraph, lines 8-10 of Maatman.

Besides, there is the difference in cellular distributions, that is, "H-FABP exists in the heart and the kidney but does not exist in the liver but on the other hand L-FABP exists in the liver and the kidney but does not exist in the heart", and further "(contrary to H-FABP) L-FABP exists topically in proximal tubules in human", which will be understood from the following description.

> The rat heart FABP cDNA could be demonstrated on the blot, to be present in rat heart and kidney mRNA but not in rat liver mRNA (Fig. 6). The blot ... showed the presence of liver FABP mRNA in both liver and kidney mRNA, but not in

> heart mRNA (Fig. 6). Page 289, left column, 2nd paragraph, lines 1-6, and Fig. 6 of Maatman.
>
> The cellular distribution of the heart-type FABP is similar in rat kidney to the previously found in human kidney [7]. The liver-type FABP, however, is restricted to the proximal convoluted and straight tubules in human kidney [7]. Page 288, right column, 1st paragraph, lines 7-11 of Maatman.
>
> Based on the RT-PCR and hybridization results, the content of the mRNAs of the liver and heart FABP types do not differ markedly in kidneys of male and female rats. Page 289,left column, 2nd paragraph, lines 8-10 and Fig. 6 of Maatman.

However, as the results of detection at the protein level (ELISA test) reveal that there was difference in the ratio of H-FABP and L-FABP as follows:

> ELISA showed low amounts of liver-type FABP in rat kidney cytosol (Table 2). The concentrations are much lower than those of the heart-type FABP, and the ratio of liver and heart-type FABPs differs considerably from that in man. Page 288, left column, 2nd paragraph, lines 1-4 and Table 2 of Maatman.

As is evident from the above explanation, according to the description of Maatman, H-FABP is neither similar nor equivalent to L-FABP, but rather, it is clearly described that both are different types of FABP. The Examiner must view the reference as a whole and cannot simply conclude that L-FABP and H-FABP are similar/equivalent by picking and choosing selected passages favorable to his interpretation. This amounts to hindsight reconstruction.

A similar result is borne out if this is viewed from the standpoint of Gorski. In Gorski the FABP (H-FABP) is one contained in the heart and to be released from the heart, as is clear from the following description:

> Heart and skeletal muscles contain the same type of FABP [referred to as heart-type (H)-FABP][1,2]...FABP is released from the heart early after the onset of infarction, whereafter its plasma concentration increases manyfold [3-6] (page 193, right column, lines 8 –19 of Gorski.

And, H-FABP is known to be contained in the heart. However, L-FABP is known not to be contained in the heart.

The above has been well known in this field, as mentioned also in Van Nieuwenhoven et al." Lipids, vol. 31, Suppl., pp. S223-S227, 1996, Table 2 etc. (filed as Exhibit 2 with Reply under 37 CFR §1.111 dated July 22, 2003) and Veerkamp et al., Prog. Lipid Res., Vol. 34, No.1, pp.17 -52, 1995, Table 3, etc. (filed as Exhibit 3 with Reply Under 37 C.F.R. § 1.111 dated July 22, 2003). The Van Nieuvenhoven et al. reference corresponds to reference [1] in Gorski.

Similar description is also found in Maatman, page 289, left column, 2nd paragraph, lines 1-6 as mentioned above.

Thus, a person skilled in the art understands the difference that H-FABP exists in the heart but on the other hand, that L-FABP does not exist in the heart. Thus, the H-FABP disclosed in Gorski (existing in the heart) is clearly distinguished from L-FABP (not existing in the heart). Thus, the two FABPs are neither similar nor equivalent.

Accordingly, from the viewpoint of both Maatman and Gorski, H-FABP and L-FABP are neither similar nor equivalent. Thus, the two FABPs are lacking in similarity/equivalence such that H-FABP would not be replaced by L-FABP. Further, Declarative evidence has been presented which illustrates the non-equivalence of L-FABP and H-FABP, which evidence is discussed below.

(iii) Maatman contains speculation only concerning L-FABP and prevention of nephrotoxicity.

Maatman contains the following discussion:

> We can only *speculate* on the physiological relevance of the two FABP types in kidney. The liver-type FABP binds various ligands and may be involved in the

> renal excretion of exogeneous and endogeneous metabolites. The liver-type FABP also binds some drugs [2,3], and may in this way prevent nephrotoxicity. (emphasis added) Page 289, right column, lines 4-10 of Maatman.

As is mentioned by the authors themselves as "We can only speculate", this description is mere speculation. Further, the speculation is only as to physiological relevance/function of FABP, but never suggests or discloses the diagnosis of kidney disease.

The Examiner has repeatedly referred to the description of "The liver-type FABP also binds some drugs, and may in this way prevent nephrotoxicity" of Maatman, however, Appellants submit that this is not definitive.

First, it should be noted, "nephrotoxicity"* is the property owned by the drug itself but not the "kidney disease" per se. Note *: According to a dictionary, "nephrotoxicity" means the quality of being toxic or destructive to kidney cells. (See attached copy of Dorland's Illustrated Medical Dictionary 27th edition, p. 1108).

Second, there is no common knowledge that markers for diagnosis of diseases are mostly a substance having an activity of preventing the disease. On the other hand, it is usually considered that even though a biological material "X" can prevent a disease, it is not said that the material "X" can be used for diagnosis of the disease, in other words, there is no direct relation between the activity for preventing a diseases and the diagnosis of the disease.

Accordingly, the speculation of Maatman (that is, merely speculation of a possibility for prevention of nephrotoxicity) is unrelated to a suggestion of using L-FABP for diagnosis of kidney disease. The speculation thus cannot constitute motivation for combining Maatman with Gorski nor for replacing H-FABP with L-FABP.

Obviousness must be predicted on something more than it would be obvious "to try" the particular class of solvent recited in the claims or the possibility it will be considered in the future, having been neglected in the past. Ex parte Argabright et al., 161 U.S.P.Q. 703 (POBA 1967). This is not altered due to KSR International, discussed above. However, this is exactly what the Examiner is contending. In the present instance, evidence exists that H-FABP and L-FABP are not equivalent. Nonetheless, the Examiner asserts that the mere recitation of these two FABPs provides motivation to replace the H-FABP of Gorski with the L-FABP of Maatman.

(iv) Gorski includes negative disclosure concerning the possibility of the application of H-FABP to diagnosis of kidney disease.

Gorski discloses that "plasma FABP [H-FABP] concentration is markedly increased in patients with renal failure" (page 194, right column, 2nd paragraph, lines 1-6 and Table 1 of Gorski). On the other hand, Gorski also mentions a detailed analysis of the results shown in Table 1 as follows:

> Neither plasma FABP nor plasma myoglobin concentrations showed a correlation with the period of dialysis or urea or creatinine concentration in plasma. Page 194, right column, 1st paragraph, lines 10-14 and Table 1 of Gorski.

That is, based on the experimental results that plasma creatinine and urea decreased after dialysis in comparison with those before dialysis, but H-FABP did not show such change in the same tests, Gorski states that there was no correlation between FABP (H-FABP) and creatinine or urea.

Creatinine and urea are generally used as markers for diagnosis of kidney disease. Accordingly, the above description of Gorski would be interpreted by one of skill to mean that H-FABP cannot be used like creatinine or urea which are markers for kidney disease. That is, the

description of Gorski represents a negative teaching concerning the application of Gorski's FABP (H-FABP) to the diagnosis of kidney disease.

With such a negative description (teach away), those of skill in the art would never expect that Gorski's FABP may be successfully applicable to the diagnosis of kidney disease even though the skilled person might have understood that plasma FABP increased in kidney failure.

(v) By replacing the H-FABP of Gorski with another molecule, there is no reasonable expectation of success in obtaining the same results.

As mentioned above, Gorski discloses that "plasma FABP [H-FABP] concentration is markedly increased in patients with renal failure". Gorski also explain that the clearance in kidney is different between H-FABP and myoglobin as follows:

> These findings suggest that the kidneys play a more dominant role in the clearance of plasma FABP than of myoglobin. Page 194, right column, 2nd paragraph, lines 21-24 of Gorski.

It is noted that FABP [H-FABP] and myoglobin are considered to be markers for heart disease (myocardial infarction) and a low-molecular-mass protein in Gorski as follows:

> ... these proteins have similar molecular masses (15 and 18kDa, respectively) and show a similar plasma release curve in patients with acute myocardial infarction and normal renal function. Page 194, right column, 2nd column, lines 16-21 of Gorski.

It is thus mentioned that two such similar molecules are still different in the clearance in kidney.

Accordingly, a person skilled in the art will certainly question whether the same results would be obtained by replacing H-FABP of Gorski with another molecule. In fact, the skilled person would have assumed that the H-FABP could not be replaced with other molecule with a reasonable expectation of success.

(vi) There exist a large distance between the present invention and the cited references.

From the above various explanations, Appellants submit that the present invention is unobvious over the cited references. However, to further reinforce this point Appellants take this opportunity to explain just how far apart the present invention is from the cited art.

In order to reach to the present invention from the cited references, it is required at least the following two steps i) and ii).

i) Change the direction to diagnosis of kidney disease from diagnosis of heart disease (myocardial infarction) in Gorski, and further,

ii) The H-FABP of Gorski must be replaced with the L-FABP of Maatman.

However, it is not easy to achieve the above steps i) and ii) as is explained below.

(a) Difficulty of the change in direction to diagnosis of "kidney disease"

Gorski aim at the diagnosis of heart disease (the diagnosis of heart disease by H-FABP). It is disclosed in Gorski that H-FABP increased in kidney diseases, but this description does not teach that H-FABP can be used for diagnosis of kidney disease. Thus, it is required to change the idea to direct to "diagnosis of kidney disease."

However, as mentioned above, Gorski includes a negative description (teaching away) with respect to the possibility of the application of H-FABP to the diagnosis of kidney disease. It would thus be very difficult to make the change to direct to the diagnosis of kidney disease by overcoming such a negative teaching. When prior art teaches away from the methods used in a patent, it is relevant and persuasive evidence of the non-obviousness of the patented matter. See Tights, Inc. v. Acme-McCrary Corp., 541 F.2d 1047, 1059, 191 USPQ 305, 312-313 (4th Cir.), cert. denied, 429 U.S. 980, 192 USPQ 64 (1976) ("the fact that the defendants' prior art

references lead away from [the patent in suit], and that even unusually skilled artisans in the field corroborated the negative teachings, is both relevant and persuasive as to the question of non-obviousness"); W. L. Gore & Associates, Inc. v. Carlisle Corp., 529 F.2d 614, 619, 189 USPQ 129, 133 (3d Cir., 1976); Shaw v. E.B. & A.C. Whiting Co., 417 F.2d 1097, 1104, 163 USPQ 580, 585-586 (2d Cir. 1969), cert. denied, 397 U.S. 1076, 165 USPQ 417 (1970).

(b) Difficulty of replacement of H-FABP of Gorski with L-FABP of Maatman.

First, the H-FABP in Gorski, and L-FABP are not similar and/or equivalent to each other and it is not easy to replace to each other. Further there is no motivation to replace both, as is explained in the above (i) to (iii).

Second, a person skilled in the art would never expect that the H-FABP of Gorski could be easily replaced with another molecule successfully as is explained in the above (iv).

Accordingly, Appellants submit that it would be quite unlikely to replace H-FABP of Gorski with the L-FABP of Maatman.

To summarize, Appellants submit that it will be not easy to accomplish both of the above step i) and ii), and unless at least these steps i) and ii) are done, the present invention cannot be reached. Accordingly, the present invention cannot be considered obvious over the combination of Gorski with Maatman and Simon.

(vii) Declarations under 37 C.F.R. § 1.132 have been submitted which negate or rebut any hypothetical *prima facie* case of obviousness

Appellants have previously provided two Declarations under 37 C.F.R. §1.132. One of these Declarations was submitted with the Reply filed on May 12, 2005 and the other was submitted with the Reply filed on March 20, 2006. These Declarations will be discussed in turn:

(a) Declaration filed May 12, 2005

The Declaration filed on May 12, 2005, copy attached, clearly explains that the present invention satisfies a long-felt need in the art. This need was recognized, persistent, and not solved by others until the present invention. For this reason alone, the Examiner's alleged *prima facie* case of obviousness is moot and should be withdrawn.

The need for a method for the diagnosis or prognosis of kidney disease in a human is a persistent one that has been recognized by those of ordinary skill in the art. In fact, the American Heart Association Science Advisory and Coordinating Committee has stated that kidney disease is a worldwide public health problem. In the United States, for example, the number of individuals with kidney failure treated with dialysis and kidney transplantation exceeded 320,000 in 1998, and continues to increase. The Declaration goes on to explain that at the present time, the prevalence of patients in the early stages of chronic kidney disease is even greater.

The Declaration explains that prior to the present invention, no sufficient technology for the diagnosis/prognosis of kidney disease had been developed. Authority cited in the Declaration explains that the identification of patients in the early stages of chronic kidney disease is critical to good patient outcome; however, that under the current state of the art (prior to the present invention), most patients are referred to nephrologists too late in the course of their renal disease for improvement. As a consequence of these late referrals to nephrologists, there is a significant increase in patient morbidity, mortality and resource utilization.

It is therefore apparent that such dire consequences would be avoidable if an adequate method for the diagnosis/prognosis of kidney disease existed. In this regard, the cited authority expressly blames the insensitivity of current screening tools for causing late referrals. Given this,

it is clear and recognized that there exists a persistent need in the art for a method for the diagnosis or prognosis of kidney disease in a human. Thus, the current development of a more practical screening tool (i.e., method for diagnosis/prognosis of kidney disease) represents a significant advance in the art.

The long-felt need in the art for a method for the diagnosis or prognosis of kidney disease in a human was not satisfied by others before the presently claimed invention was invented by Appellants, and, the present invention is clearly effective, useful and superior over existing methods. Thus, the Board is requested to reverse the Examiner's rejection based upon Gorski, Maatman and Simon.

(b) Declaration filed March 20, 2006

The Declaration filed on March 20, 2006, copy attached, clearly explains that H-FABP and L-FABP are neither similar nor equivalent, and that the present invention achieves unexpectedly superior results compared to the method utilizing H-FABP in the prior art. This Declaration is important since even if the Examiner has hypothetically established a *prima facie* case of obviousness, a point not conceded by Appellants, the presently claimed subject matter still achieves unexpectedly superior results compared to the prior art. For instance, even assuming that H-FABP and L-FABP are suggested by the prior art and should be tested for when attempting to diagnose or prognose kidney disease, the results achieved are not equivalent.

Review of the March 20, 2006 Declaration reveals that urinary levels of L-FABP are much more elevated over time after administration of contrast media compared to urinary levels of H-FABP. This is evident from a review of the data. For instance, the mean values with

standard deviation (S.D.) are shown in Table 1 of the Declaration. The same data is shown graphically in Figure 1 (in Figure 1, the upper bar only is described for S.D.).

Thus, even if the elevation of both of H-FABP and L-FABP in renal disease would be suggested or expected (which Appellants do not believe is true) it would still be unexpected that the levels of L-FABP would be so significantly elevated compared to levels of H-FABP. Therefore, even assuming the Examiner has established a *prima facie* case of obviousness, a point not conceded, the unexpectedly superior results demonstrated in the March 20, 2006 Declaration render any hypothetical *prima facie* case of obviousness moot.

In view of the foregoing, Appellants respectfully submit that there are significant patentable distinctions between present claim 16 and the teachings of Gorski when taken in view of Maatman and Simon.

Dependent Claim 4

Claim 4 depends upon claim 16, and requires that the specimen (which may be either kidney tissue or urine in claim 16) is urine. All of the above distinctions with respect to the cited art remain. Claim 4 is additionally distinct in requiring that the specimen be a urine specimen.

This represents an additional distinction from the cited art and based upon the above arguments and the attached evidence, including the Declarations under 37 C.F.R. § 1.132, the Board is requested to reverse the Examiner's rejection of claim 4 based upon Gorski, Maatman and Simon.

Dependent Claim 2

Claim 2 depends upon claim 4, and requires that the L-FABP is derived from kidney tissue. All of the above distinctions with respect to the cited art (including those for claim 16 and claim 4) remain. Claim 2 presents an additional distinction.

Therefore, based upon the above arguments and the attached evidence, including the Declarations under 37 C.F.R. § 1.132, the Board is requested to reverse the Examiner's rejection of claim 2 based upon Gorski, Maatman and Simon.

Dependent Claim 6

Claim 6 depends upon claim 4 and requires an additional step to the method of claim 16. This step is one of comparing the test result with that of a control specimen, the control specimen being collected from a human having normal kidney tissue, or collected from a human having the same kidney disease but showing different symptoms or different progress. All of the above distinctions with respect to the cited art (including those for claim 16 and claim 4) remain. Claim 6 presents an additional distinction concerning the nature of a comparison for evaluating the test result with a control specimen.

Therefore, based upon the above arguments and the attached evidence, including the Declarations under 37 C.F.R. § 1.132, the Board is requested to reverse the Examiner's rejection of claim 6 based upon Gorski, Maatman and Simon.

Dependent Claim 17

Claim 17 depends upon claim 4 and further defines the way in which step (b) of claim 16 is carried out. Specifically, claim 17 requires that step (b) be carried out by: (i) contacting the

specimen with an antibody specifically binding to L-FABP; (ii) separating unbound antibody from the antibody bound to the protein; and (iii) detecting the antibody bound to the protein. Each of the above distinctions remains.

Therefore, based upon the above arguments and the attached evidence, including the Declarations under 37 C.F.R. § 1.132, the Board is requested to reverse the Examiner's rejection of claim 17 based upon Gorski, Maatman and Simon.

Dependent Claim 18

Claim 18 depends upon claim 4 and requires that the existing level of L-FABP in the specimen is diagnostic or prognostic of the kidney disease. Each of the above distinctions (discussed with respect to claim 16 and claim 4) remain. The prior art fails to recognize that existing levels of L-FABP may be diagnostic or prognostic of the kidney disease.

Therefore, based upon the above arguments and the attached evidence, including the Declarations under 37 C.F.R. § 1.132, the Board is requested to reverse the Examiner's rejection of claim 18 based upon Gorski, Maatman and Simon.

Dependent Claims 22 and 23

Claim 22 depends upon claim 4 and requires that the kidney disease is a disease selected from the group consisting of diabetic nephropathy, glomerulonephritis, nephrotic syndrome, focal glomerulosclerosis, immune complex nephropathy, lupus nephritis, drug-induced renal injury, renal insufficiency and kidney graft rejection. Claim 23 depends upon claim 22 and further requires that the immune complex nephropathy is selected from the group consisting of IgA nephropathy and membranous nephropathy. Each of the above distinctions (discussed with

respect to claim 16 and claim 4) remain. The cited art is silent concerning the specific types of kidney disease delineated in claims 22 and 23.

Therefore, based upon the above arguments and the attached evidence, including the Declarations under 37 C.F.R. § 1.132, the Board is requested to reverse the Examiner's rejection of claims 22 and 23 based upon Gorski, Maatman and Simon.

Independent Claim 24

Claim 24 is the second independent claim at issue other than claim 16. The only difference between claim 16 and claim 24 is that while claim 16 relates to "diagnosis or prognosis of a kidney disease", claim 24 relates to "diagnosing the progression of kidney disease." Therefore, claim 24 allows for the monitoring of the progression of kidney disease.

Each of the above distinctions discussed under the subheading for claim 16 are equally relevant to claim 24. However, due to the difference discussed above, claim 24 provides an additional distinction over the cited art in that there is no suggestion of "diagnosing the progression" of a given kidney disease by detecting L-FABP in the specimen.

Therefore, based upon the above arguments and the attached evidence, including the Declarations under 37 C.F.R. § 1.132, the Board is requested to reverse the Examiner's rejection of claim 24 based upon Gorski taken in view of Maatman and Simon.

Dependent Claim 27

Claim 27 depends upon claim 24, and requires that the specimen (which may be either kidney tissue or urine in claim 24) is urine. All of the above distinctions with respect to the cited art remain. Claim 27 is additionally distinct in requiring that the specimen be a urine specimen.

This represents an additional distinction from the cited art and based upon the above arguments and the attached evidence, including the Declarations under 37 C.F.R. § 1.132, the Board is requested to reverse the Examiner's rejection of claim 27 based upon Gorski, Maatman and Simon.

(2) Claim 9 is non-obvious over the references of Gorski, Maatman, Simon and Kimura

The Examiner has alleged that claim 9, which depends upon claim 17 (discussed above), is obvious in light of Gorski taken in view of Maatman, Simon and Kimura. The Examiner relies on Gorski, Maatman and Simon as applied in the rejection discussed under heading (1) above, but acknowledges that these references fail to suggest that L-FABP is found in the proximal tubule of the kidney and does not cross-react with H-FABP.

The Examiner utilizes the additional secondary reference of Kimura to reject claim 9. Claim 9 depends upon claim 17 (discussed above) and additionally requires that the antibody specifically binding to L-FABP is an antibody that does not cross-react with H-FABP (heart muscle-type fatty acid binding protein). All of the above distinctions discussed under item (1) above remain, and, claim 9 represents additional distinction.

Kimura merely discloses H-FABP and a protein derived from modification of α2u-globulin, existing kidney tissue of male rat, and detected by specific antibodies. As such, claim 9 remains patentable for the same reasons as claim 17 is patentable. Further, there exists no motivation to further modify the method of claim 16 (upon which claim 9 depends, indirectly through claim 17) based upon the proteins or antibodies taught by Kimura.

For these reasons, the Board is requested to reverse the Examiner's rejection of claim 9 based upon Gorski, Maatman, Simon and Kimura.

(3) Claims 19 and 21 are non-obvious over the references of Gorski, Maatman, Simon and Galaske

The Examiner has cited Gorski, Maatman and Simon for the same reasons as discussed under item (1) above, but acknowledges that these references fail to suggest a detection system involving a chronic renal disease and further monitoring the specimen collection at various time intervals. Therefore, the Examiner utilizes the additional secondary reference of Galaske to rejection claims 19 and 21.

Claim 19 depends upon claim 4 (discussed above) and requires an additional step of comparing the test result of the specimen with a different specimen collected from the same human at different stage, and examining the change with the lapse of time. Claim 21 depends upon claim 4 and requires that the kidney disease is a chronic renal disease.

All of the above distinctions discussed under item (1) above remain. However, claims 19 and 21 represent additional distinctions. Although Galaske discusses glomerular filtration and tubular uptake of plasma proteins in the acute heterologous phase of an anti-GMB nephritis model, this fails to suggest to those of skill in the art that detection of L-FABP can be used to diagnose or prognose kidney disease. As such, claims 19 and 21 remain patentable for the same reasons as claim 4 is patentable. Further, there exists no motivation to further modify the method of claim 16 (upon which claims 19 and 21 depend, indirectly through claim 4) based upon a nephritis model taught by Galaske.

For these additional reasons, the Board is requested to reverse the Examiner's rejection of claims 19 and 21 based upon Gorski, Maatman, Simon and Galaske.

VIII. CONCLUSION

Appellants have demonstrated that the Examiner has failed to successfully allege that the rejected claims are *prima facie* obvious. For the reasons advanced above, it is respectfully submitted that all claims in this application are allowable. Thus, favorable reconsideration and reversal of the Examiner's rejections under 35 U.S.C. §103(a), by the Honorable Board of Patent Appeals and Interferences, are respectfully solicited.

The required Appeal Brief fee in the amount of $500.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Dated: JUN - 8 2007

Respectfully submitted,

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Appendices: 1) CLAIMS APPENDIX
2) EVIDENCE APPENDIX
3) RELATED PROCEEDINGS APPENDIX

Attachments: As outlined in Evidence Appendix

VIII. CLAIMS APPENDIX

1. (Cancelled).

2. (Previously Presented) The method according to claim 4, wherein the liver-type fatty acid binding protein is derived from kidney tissue.

3. (Cancelled).

4. (Previously Presented) The method according to claim 16, wherein the specimen is urine.

5. (Cancelled).

6. (Previously Presented) The method according to claim 4, which further comprises a step of comparing the test result with that of a control specimen, said control specimen being collected from a human having normal kidney tissue, or collected from a human having the same kidney disease but showing different symptoms or different progress.

7-8. (Cancelled).

9. (Previously Presented) The method according to claim 17, wherein the antibody specifically binding to the liver-type fatty acid binding protein is an antibody that does not cross-react with a heart muscle-type fatty acid binding protein.

10-15. (Cancelled).

16. (Previously Presented) A method for diagnosis or prognosis of a kidney disease in human, which comprises the steps of:

(a) preparing a specimen collected from a human;

(b) detecting liver-type fatty acid binding protein contained in said specimen; and

(c) diagnosing or prognosing the kidney disease based on the test result of the detection in (b), wherein said specimen is kidney tissue or urine.

17. (Previously Presented) The method according to claim 4, wherein the step (b) is carried out by

(i) contacting the specimen with an antibody specifically binding to liver-type fatty acid binding protein;

(ii) separating unbound antibody from the antibody bound to said protein; and

(iii) detecting the antibody bound to said protein.

18. (Previously Presented) The method according to claim 4, wherein the existing level of liver-type fatty acid binding protein in the specimen is diagnostic or prognostic of the kidney disease.

19. (Previously Presented) The method according to claim 4, which further comprises the step of comparing the test result of the specimen with a different specimen collected from the same human at different stage, and examining the change with the lapse of time.

20. (Cancelled).

21. (Previously Presented) The method according to claim 4, wherein the kidney disease is a chronic renal disease.

22. (Previously Presented) The method according to claim 4, wherein the kidney disease is a disease selected from the group consisting of diabetic nephropathy, glomerulonephritis, nephrotic syndrome, focal glomerulosclerosis, immune complex nephropathy, lupus nephritis, drug-induced renal injury, renal insufficiency and kidney graft rejection.

23. (Previously Presented) The method according to claim 22, wherein the immune complex nephropathy is selected from the group consisting of IgA nephropathy and membranous nephropathy.

24. (Previously Presented) A method for diagnosing the progression of kidney disease in a patient suffering therefrom, comprising the steps of:

(a) preparing a specimen collected from said patient;

(b) assaying for liver-type fatty acid binding protein contained in said specimen; and

(c) diagnosing the progression of the kidney disease based on the test result of the detection in (b), wherein said specimen is kidney tissue or urine.

25-26. (Cancelled).

27. (Previously Presented) The method according to claim 24, wherein the specimen is urine.

IX. EVIDENCE APPENDIX

EVIDENCE	STATEMENT
Van Nieuwenhoven et al., Lipids, vol. 31, Suppl., pp. S223-S227, 1996, Table 2 etc.	This reference was submitted as Exhibit 2 with the Reply under 37 CFR §1.111 dated July 22, 2003. This reference was also discussed in the Reply filed May 12, 2005 and March 20, 2006.
Veerkamp et al., Prog. Lipid Res., Vol. 34, No.1, pp.17-52, 1995.	This reference was submitted as Exhibit 3 with the Reply under 37 C.F.R. § 1.111 dated July 22, 2003. This reference was also discussed in the Reply filed May 12, 2005 and March 20, 2006.
Dorland's Illustrated Medical Dictionary 27th edition, p. 1108.	This excerpted reference was filed as an attachment to the Reply under 37 C.F.R. § 1.116 dated May 12, 2005.
Declaration under 37 C.F.R. § 1.132 (executed May 10, 2005) including exhibits A, B, C and D.	This Declaration (along with Exhibits A, B, C, and D) was submitted with the Reply under 37 C.F.R. § 1.116 dated May 12, 2005.
Declaration under 37 C.F.R. § 1.132 (executed March 14, 2006).	This Declaration was submitted with the Reply under 37 C.F.R. § 1.111 dated March 20, 2006.
Wodzig et al., Ann. Clin. Biochem., Vol. 34, pp. 263-268, 1997.	This reference was submitted as Exhibit 1 with the Reply under 37 CFR §1.111 dated July 22, 2003.
Kamijo et al., J. Lab. Clin. Med., Vol. 143, No. 1, pp. 23-30, 2004	This reference was discussed in the Declaration under 37 CFR §1.132 (executed May 10, 2005)(page 9 to 10). This reference was also submitted as Exhibit 1 with the Reply under 37 CFR §1.111 dated Oct. 14, 2004.

X. RELATED PROCEEDINGS APPENDIX

Not Applicable – None

Membrane-Associated and Cytoplasmic Fatty Acid-Binding Proteins

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ABSTRACT: A number of cellular fatty acid-binding proteins are being implicated in the uptake and intracellular transport of long-chain fatty acids by parenchymal cells. Having been a topic of research for more than 20 years, cytoplasmic fatty acid-binding proteins now are assigned various pivotal functions in intracellular fatty acid transport and metabolism. More recently several membrane-associated fatty acid-binding proteins have been identified and these proteins are thought to function in the transmembrane transport of fatty acids. In this review, a short summary is provided of the latest developments in this research area.

Lipids 31, S-223–S-227 (1996).

Long-chain fatty acids (FA) are important compounds serving as substrates for energy-production and formation of phospholipids (1). Moreover, they can participate in signal transduction pathways (2–4). Adipose tissue takes up large amounts of FA from blood plasma for storage as intracellular triacylglycerols. In contrast, oxidative skeletal muscles and especially the heart take up FA as fuel for contractile processes. Since FA are poorly soluble in water the rapid metabolism of these FA would probably not be possible without special binding proteins, which dramatically increase the solubility of these compounds in aqueous environments. In blood and interstitium, FA are mainly bound to albumin, keeping the unbound FA concentrations extremely low (<10 nM) (5). Intracellularly, FA are most likely bound to specific cytoplasmic fatty acid-binding proteins (FABP) (6–10). More recently, membrane-associated proteins have been hypothesized to function in the cellular uptake of FA. These membrane and cytoplasmic proteins will be discussed in more detail below.

Membrane-associated FABP. The mechanism of transmembrane transport of FA into parenchymal cells has been a matter of debate for several years. Some investigators favor a mechanism in which FA cross the membrane by simple diffusion (11,12). Others found saturable uptake of FA in several cell-types, a process that could be inhibited by FA analogues, indicating that membrane-associated proteins are involved (13). Subsequently, by using various techniques, a number of such membrane proteins have been identified (Table 1). The first membrane protein described to be involved in the uptake of FA is a 40 kDa protein present in the plasma membrane of rat liver cells (14). This protein, $FABP_{PM}$, was subsequently also found in intestine (15), and heart (16). The protein seems closely related, if not identical, to mitochondrial aspartate aminotransferase (17). $FABP_{PM}$ is not an integral membrane protein but has been shown to possess affinity for membranes (17).

In 1987, Fujii *et al.* (18,19) described another membrane protein with high affinity for FA, which was designated fatty acid receptor (FAR). This protein of about 60 kDa was found to be present in kidney and heart. The third membrane protein putatively involved in FA uptake, identified in adipocytes by Harmon and co-workers (20) and recently cloned by Abumrad *et al.* (21), is an 88 kDa protein called fatty acid translocase (FAT). FAT from rat was found to be highly homologous (85%) to the human leucocyte differentiation antigen CD36, a receptor protein present among others on monocytes and platelets, and thought to be involved in adhesion phenomena and intracellular signalling (for review on CD36 see ref. 22). Comparison of rat FAT (21) and the recently cloned mouse CD36 (23) revealed an amino acid identity of 93%, which strongly suggests that these proteins are species homologues. Recent investigations showed that CD36 is an integral membrane protein with one transmembrane region (24).

In adipocytes, Trigatti *et al.* (25) found another membrane protein implicated in the transmembrane transport of FA. This 22 kDa protein was identified by photoaffinity labelling with a FA analogue. The final membrane protein hypothesized to augment cellular FA uptake that has been found up till now, is called fatty acid transport protein (FATP) (26). A functional approach using expression cloning was followed to identify this 63 kDa protein in adipocytes. FATP was shown to be an integral membrane protein present in the plasma membrane of several tissues, and has been predicted to have several membrane spanning domains (26).

The precise functions of the FA-binding membrane-associated proteins in the transport of FA across cellular membranes are still unclear. Some of the possible mechanisms by which membrane proteins could be involved in this process

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Abbreviations: FA, long-chain fatty acids; FABP, fatty acid-binding protein; FAR, fatty acid receptor; FAT, fatty acid translocase; FATP, fatty acid transport protein.

TABLE 1
Membrane-Associated Fatty Acid-Binding Proteins (FABP)

Protein	Molecular mass (kDa)	Major occurrence	Reference
Membrane FABP	22	Adipose tissue	25
$FABP_{PM}$	40	Liver, heart, adipose tissue, intestine	15,16
FA receptor	56–60	Heart, kidney	18,19
FA transport protein	63	Adipose tissue, heart, skeletal muscle	26
FA translocase	88	Adipose tissue, heart, skeletal muscle	20,21

are shown in Figure 1. These proteins might function as a translocator, but it is also possible that they represent acceptors for FA released from albumin and that FA subsequently cross the plasma membrane by diffusion through the phospholipid bilayer. Albumin binding proteins have been described which could play a role in this mechanism (27). Furthermore, there might be a direct interaction between a membrane-associated FA-binding protein and (extracellular) albumin and/or (intracellular) FABP. Such interactions would ensure a controlled uptake mechanism in which FA remain protein-bound. Further characterization of the FA-binding membrane proteins will provide a better understanding of the complex transmembrane transport mechanisms of FA.

Intracellular FABP. Cytoplasmic FABP belong to a single gene family of intracellular lipid binding proteins of 14–15 kDa, capable of binding hydrophobic ligands with high affinity (28). The mammalian cytoplasmic lipid-binding proteins are listed in Table 2. Since the discovery of FABP in 1972 by Ockner and co-workers (29), nine different FABP, including the recently discovered testicular 15 kDa protein (T-LBP) (30,31), have been identified (Table 2). All lipid binding proteins are composed of two α-helices and 10 anti-parallel β-strands, organized in two β-sheets, thereby forming a clam shell-like structure (8). The lipid ligand is bound inside the molecule by interaction with specific amino acid residues within the binding pocket of the lipid binding protein (8). A recent investigation by Richieri *et al.* (32) suggests that the FABP have a higher affinity for the binding of FA than found in previous studies (7,9,10) with the dissociation constants (Kd) ranging from 2 to 1000 nM, depending on type of FABP and FA (32). A striking feature of the FABP is their relative abundance in tissues with active FA metabolism.

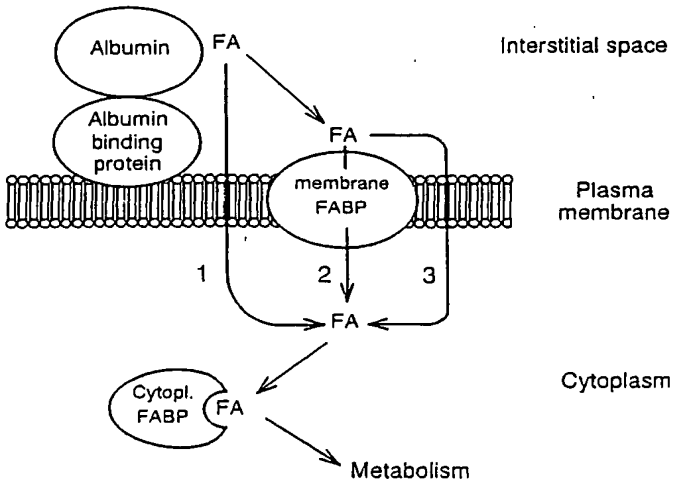

FIG. 1. Schematic presentation of the mechanism of cellular fatty acid uptake and the possible role of membrane-associated fatty acid-binding proteins in this process. Transmembrane transport could take place without the involvement of membrane proteins (1), or with a membrane protein acting as a true translocator (2), or as an acceptor for fatty acids thus, creating a steeper transmembrane gradient (3). FA, long-chain fatty acid; membrane FABP, any membrane-associated fatty acid-binding protein (see Table 1); cytopl. FABP, any cytoplasmic FABP (see Table 2).

A number of biological roles have been ascribed to the FABP: (i) Facilitation of the transport of FA to their intracellular sites of utilization. Indirect evidence was provided in studies showing a correlation between FA-utilization and FABP-content (33,34) and by showing that *in vivo* FA are bound to FABP (35,36). Furthermore, *in vitro* studies showed an increase in FA transport from isolated mitochondria to artificial phospholipid vesicles in the presence of H-, or L-FABP (37). Theoretical studies also support a role for FABP in FA-transport in cardiomyocytes (38). More direct evidence was found by transfection studies in which cells transfected with H-, L-, or A-FABP showed increased FA-uptake rates (39-41). Finally, a recent report showed that a single amino acid substitution (Ala54 -> Thr54) in human I-FABP, as occurs in some Pima Indians, causes a two-fold greater affinity for FA. This was associated with increased fat oxidation rates and with insulin resistance, also suggesting a role for I-FABP in cellular FA-transport (42). (ii) Prevention of local high FA concentrations and thereby protecting the cell against detrimental effects of FA, for instance in the ischemic heart (43). (iii) By modulating hydrophobic ligand metabolism, FABP can influence important cellular events like mitogenesis (44) and FA-mediated signal transduction pathways (3,7). Recently, a small fraction of H-FABP in rat heart and mammary gland was found to be phosphorylated upon stimulation with insulin, and it was suggested that H-FABP might play a role in signal transduction downstream from the insulin receptor (45,46). (iv) Inhibition of growth and induction of differentiation of cultured mammary epithelial cells has been reported as an extracellular function of mammary derived growth in-

TABLE 2
Mammalian Cytoplasmic Lipid-Binding Proteins[a]

Protein	Current designation	Other designations	Ligand	Major occurrence
Heart FABP	H-FABP	MDGI	FA	Heart, skeletal muscle, smooth muscle, brain, kidney, mammary gland
Liver FABP	L-FABP	Z-protein	FA, heme, bilirubin, prostaglandins	Liver, small intestine, kidney
Intestinal FABP	I-FABP		FA	Small intestine
Adipocyte lipid-binding protein	A-LBP	A-FABP, aP2	FA, retinoic acid	Adipose tissue
Epidermal FABP	E-FABP		FA	Epidermis
Brain FABP	B-FABP		FA	Nervous system
Myelin FABP	M-FABP	Myelin-P2	FA, retinoids	Nervous system
Ileal lipid-binding protein	I-LBP	I-BABP, gastrotropin	FA, bile acids	Small intestine (ileum)
Testicular lipid-binding protein	T-LBP	PERF15	ND	Testis
Cellular retinoic acid-binding protein	cRABP		Retinoic acid	Testis, nervous system, kidney, skin
Cellular retinoic acid-binding protein II	cRABPII		Retinoic acid	Skin, adrenals
Cellular retinol-binding protein	cRBP		Retinol	Widespread expression
Cellular retinol-binding protein II	cRBPII		Retinol	Intestine

[a]Data are compiled from References 3, 8, 30, 31, and 56. ND, not determined; FA, long-chain fatty acids, other abbreviation as in Table 1.

hibitor, which is identical to H-FABP (9). (v) Induction of myocyte hypertrophy through binding with a high affinity receptor is another extracellular function recently ascribed to H-FABP (47).

Regulation of expression of FABP. The expression of A-FABP and also of FAT and FATP is upregulated during differentiation from preadipocytes to adipocytes (21,26,48), and is paralleled or preceded by an increased uptake of FA (48).

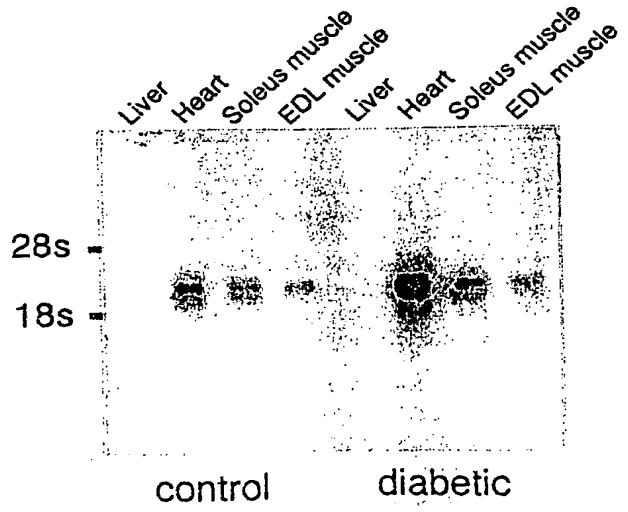

FIG. 2. Northern blot analysis of total RNA from liver, heart, soleus, and extensor digitorum longus (EDL) muscle from control and streptozotocin-induced diabetic rats using fatty acid translocase cDNA as probe.

Recently, it was shown that FA themselves can induce the expression of A-FABP in rat preadipocytes (49).

In cardiac cells, H-FABP expression increases markedly during the first few days after birth and in the weaning period (50,51). During this period, the FA oxidation capacity is also increased (52). Recently, it was shown that FAT is co-expressed with H-FABP in rat muscles and that FAT expression followed a similar upregulation in heart during development, indicating related biological functions (53).

Diabetes is a disease in which several organs, like the heart, rely more on FA oxidation as their major energy source. In streptozotocin-induced diabetic animals, a decrease of A-FABP expression (54) and an increase in H-FABP expression (55) have been shown. These results indicate that insulin deficiency can influence FABP expression levels. Preliminary results from our laboratory show that FAT is also upregulated in rat muscles in streptozotocin-induced diabetic rats (Fig. 2). These results suggest that altered FA-handling of cells can influence FABP and FAT expression.

Concluding remarks. In the last 20 years, a number of cellular proteins with affinity for FA have been identified. Among these are nine different types of intracellular 15 kDa FABP which most likely function in facilitating the *trans*-cytoplasmic transport of FA. In the last few years, more interest in the transmembrane transport of FA has resulted in the discovery of membrane proteins that might be involved in this process. Now that the genes of most of these proteins have been cloned, molecular biological techniques can be applied to manipulate the cellular content and binding activity of

these proteins. This might provide a better understanding of their role in the complex mechanisms by which FA are taken up and distributed in the cell.

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CYTOPLASMIC FATTY ACID-BINDING PROTEINS: THEIR STRUCTURE AND GENES

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CONTENTS

I. INTRODUCTION

Lipid-binding, -transfer or -exchange proteins are present in intra- and extracellular fluids of all organisms. They play a role in the transport or targeting of lipids in the cell or in the plasma, but may also interact directly or indirectly by modulation of the free ligand concentration with various cellular processes. Some lipid-binding proteins are rather specific, others bind various hydrophobic ligands, e.g. serum albumin, non-specific lipid transfer protein, liver fatty acid-binding protein (FABP). Lipid-binding molecules belong to several unrelated families of proteins and many molecules have not been characterized well. The structure of three families of lipid-binding proteins has been established (Table 1). Many of these proteins bind fatty acids as their main ligand, but also proteins with a quite different structure have affinity to fatty acids (Table 2). Before we come to the main subject of this review, the cytoplasmic fatty acid-binding proteins (FABPs) and the other members of the FABP family, we will first shortly discuss other proteins which bind fatty acids.

Albumin is the main transporter of free fatty acids in the blood,[318] but in the fetal blood α-fetoprotein and fetuin are also involved in fatty acid transport.[342] Serum vitamin D-binding protein has a low affinity for fatty acids.[53] The structure of the fatty acid-binding sites of albumin[319] and human α-fetoprotein[236] have been described. Lactoglobulin is the only member of the lipocalins (Table 1) which has a rather high affinity for fatty acids,[320,264] but also binds retinol.[264] Heat-shock protein (72 kDa) contains 2 molecules of nonesterified palmitic and oleic acid each per isolated dimer.[109] Glutathione *S*-transferases bind fatty acid in their regulatory domain II at the C-terminal side.[184,235] Other proteins which bind fatty acids are the membrane fatty acid-binding proteins found in *Escherichia coli*[37] and in the plasma membrane of various mammalian cell types as adipocytes, enterocytes, hepatocytes and myocytes.[262,315] We will discuss these proteins below.

TABLE 1. Members and Characteristics of Three Families of Lipid-Binding Proteins

I	II (Lipocalins)	III
Albumin	β-Lactoglobulin	FABPs (7 types)
α-Fetoprotein	Serum retinol BP	CRBPs (2 types)
Vitamin D BP	Bilin BP (insect)	CRABPs (2 types)
	α_1 Microglobulin	
	Apoliproprotein D	
	Odorant BP	
	α_{2U} Globulin	
50–70 kDa	18–20 kDa	12–15 kDa
Double loops	β-Barrel of 8 strands	β-Clam of 10 strands
Extracellular	Extracellular	Intracellular

The extracellular lipocalins show similar architectural principles as the family of intracellular binding proteins of fatty acids, retinol and retinoic acid (Table 1). Proteins of both families possess a β-barrel structure of eight and ten strands, respectively, with the first strand shared by two orthogonal sheets.[100,144,255,285] The integrity of the barrel is retained upon removal of the ligand. The lipocalins act as extracellular binding proteins for different hydrophobic ligands, among which retinol is bound by various members. Recently an enzyme, prostaglandin D synthase, was identified as a member of the lipocalin family of hydrophobic molecule transporters.[220]

The structure of intracellular glycolipid-, phospholipid-, cholesterol- and many other lipid-binding proteins have no resemblance to that of the FABPs. Intracellularly, also an acyl-CoA binding protein is present.[168,169] This molecule has a completely different structure, although the acyl chain is also bound like in FABP in a bent conformation near non-polar residues in the interior of the protein.[170] This acyl-CoA binding protein does not bind fatty acids, but has a very high affinity to acyl-CoA esters[266,267,275] and is involved in their intracellular metabolism.[169,267]

In this review we will shortly describe the membrane fatty acid-binding proteins, but the (cytoplasmic) FABPs form the main subject. Since we and others previously considered many structural and possible functional aspects of these latter molecules,[17,19,21,97–99,102,145,208,249,350,374,375] we discuss especially recent data on their gene structure and on regulation of their gene expression. Fatty acids and FABPs appear to be directly or indirectly involved in modulation of several cellular processes including gene expression.[375] The latter point will also be taken into consideration in this review. For comparison we include some related data on the other members of the FABP family, the cellular retinol-binding proteins (CRBP I and II) and the cellular retinoic acid-binding proteins (CRABP I and II).

II. FATTY ACID UPTAKE AND TRANSPORT AND FATTY ACID-BINDING PROTEINS

The mechanism by which fatty acids traverse the plasma membrane has already been a controversial point for many years.[249,374] To understand fatty acid uptake, it is essential

TABLE 2. Proteins with a High Affinity for Fatty Acids

Protein	*M* (kDa)	Other ligands	Number high binding sites	Occurrence
Albumin	68	Bilirubin, drugs	2–6	Plasma
α-Fetoprotein	70	Oestrogen	1	Fetal plasma and tissues
Fetuin	49	?	1	Fetal plasma
Vitamin D-BP	53	Vitamin D	1	Plasma
β-Lactoglobulin	35	no	1	Milk
Heat-shock protein	71	?	2	Intracellular
Plasma membrane FABP	40–85	?	1	Cellular
Cytoplasmic FABPs	15	No—various	1–2	Intracellular
Glutathione *S*-transferase	47	Bilirubin, heme, bile acid	1	Intracellular

to consider all potentially limiting steps and their implications for the process.[388] Diffusion and dissociation from albumin may limit fatty acid uptake at physiologic albumin concentration.[90,363,390] The presence of albumin receptors and their relation with fatty acid uptake is equivocal in hepatocytes.[277,388,389] but they may exist in endothelium[7] and cardiomyocytes.[260] Receptor-mediated transcytosis of albumin may play a role in the modulation of the amount of fatty acids that are delivered to the target cells and tissues.[7] Evidence for a dual-receptor model was reported for the fatty acid uptake by resting and activated lymphocytes.[367] Albumin- and alpha-foetoprotein receptors as well as plasma membrane fatty acid-binding protein would be involved.

Fatty acid transport across the plasma membrane of various cell types has been considered as a passive process by simple diffusion and lipid partition.[49,70,239,274] This transport is followed by binding to the cytoplasmic FABP and transfer to cellular organelles[249,374] (Fig. 1) or further flow along internal membranes.[302] Native fatty acids and also most covalently labeled fatty acids move rapidly across a phospholipid bilayer via the unionized form[149,150] in contrast to 12-(9-anthroyloxy)stearic acid.[165,334] The slow flip–flop rate of this fatty acid has been used to postulate the necessity of proteins for transport of fatty acids across membranes.[165] Other investigations gave also evidence for a plasma membrane fatty acid-binding protein- or carrier-mediated uptake. The fast flip–flop of unchanged fatty acids may, however, provide a simple and energy-dependent mechanism for their entry into hepatocytes, myocytes and adipocytes. In biological membranes a membrane fatty acid-binding protein may be required to sequester the fatty acids to the phospholipid bilayer, where they can undergo flip–flop.[249,374] A transmembrane transporter protein as postulated in the mitochondrial inner membrane[311] could have a function in transfer of ionized fatty acid across the membrane.[150]

In *Escherichia coli* long-chain fatty acids require for their transport across the outer membrane the presence of the FadL protein.[37] The high fatty acid-binding affinity of this

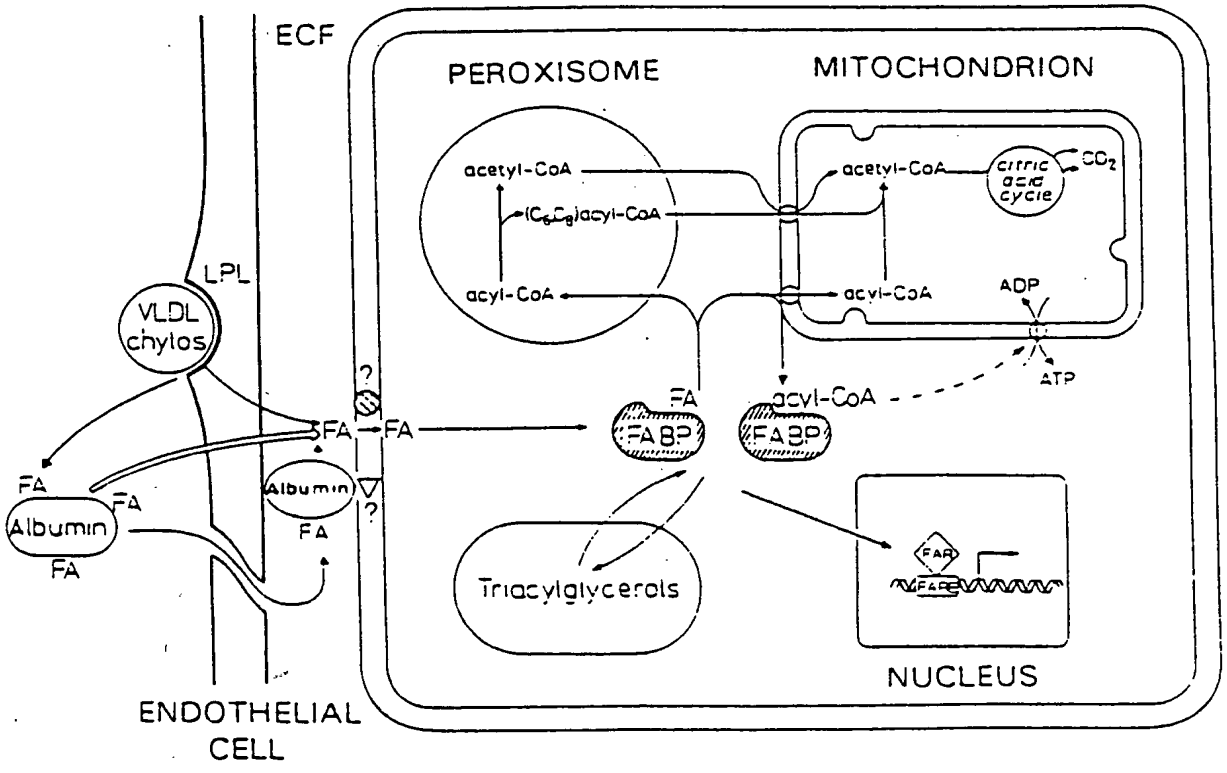

Fig. 1. Schematic representation of the fatty acid transfer from blood to cellular organelles and systems of lipid synthesis. Abbreviations: FA, fatty acid; VLDL, very low density lipoproteins; chylos, chylomicrons; ECF, extracellular fluid; FAR, putative FA receptor; FARE, putative FA receptor element.

protein could be established by photo-affinity labeling.[204] Studies on mutants of FadL demonstrated that the amino acids Phe[448], Pro[428], Val[410] and Ser[397] of the carboxyl region of this protein are involved in the binding and/or transport of fatty acid.[172,173] The ways of transport of fatty acid through the periplasmic space and across the inner membrane of the bacterium have not been defined. No evidence was found for the presence of a fatty acid-binding protein in the inner membrane of the cell envelope.[204] Recruitment of acyl-CoA synthetase to the inner membrane resulted in a 10-fold increase in the rate of fatty acid transporter.[205] This may indicate that the membrane-associated pool of fatty acids is the direct substrate for the enzyme.

The involvement of membrane fatty acid-binding proteins in fatty acid uptake into mammalian cells is based on action of inhibitors and proteases, on binding and kinetic data, on the isolation of these proteins and on the inhibition of fatty acid uptake by an antibody against such a protein.[262,315,316] The identity of these proteins is, however, rather unclear. Proteins with a molecular mass of 40–43 kDa were isolated by affinity chromatography from rat cardiac myocytes,[316,336] adipocytes,[301] jejunal microvillous membranes,[338] and liver plasma membranes.[339] The proteins appeared to be immunologically similar with a polyclonal antibody against the liver protein,[316] but not with a monoclonal antibody.[77] Antibodies against the protein from liver plasma membranes inhibited fatty acid uptake into hepatocytes, adipocytes and cardiac myocytes,[316,336,407] but not into keratinocytes in which a fatty acid uptake mechanism was found with preference for linoleic acid.[300] The surface expression of the protein is virtually identical in hepatocytes of male and female rats.[317]

The plasma membrane fatty acid-binding protein and the mitochondrial isoenzyme of aspartate aminotransferase from rat liver appeared to be related proteins,[29,341] but this was disputed.[337] Recently it was reported that fibroblasts transfected with an aspartate aminotransferase cDNA express both plasma membrane fatty acid-binding protein and saturable fatty acid uptake.[134] The 43-kDa plasma membrane fatty acid-binding protein was only expressed in differentiated adipocytes.[407] Other groups identified a plasma membrane fatty acid-binding protein of 22 kDa by photoaffinity labeling, both in undifferentiated fibroblasts and differentiated adipocytes.[359] Labeling with sulfo-*N*-succinimidyl oleate gave a plasma membrane protein with an apparent size of 85 kDa[114] in accordance with previous permeation studies.[2] Recently, the cDNA and the deduced protein structure of this rat adipocyte membrane protein implicated in fatty acid binding and/or transport were reported.[3] The sequence of the 472-amino acid (52.5 kDa) protein predicted two transmembrane segments and 10 potential N-linked glycosylation sites. The protein sequence is 85% similar with that of the glycoprotein IV (CD36) of platelets. Northern blots probed with the cDNA showed transcripts in differentiated adipocytes, heart, intestine, muscle and testis, but not in preadipocytes, liver and kidney.[3] Studies on fatty acid uptake in adipocytes with fluorescence microscopy indicated that a large fraction of transport is mediated by protein.[335] Treating adipocytes with the membrane-impermeable reagent 4,4′-diisothiocyanostilbene-2,2′-disulfonate inhibited more than 50% of the long-chain fatty acid transport. The role of the plasma membrane fatty acid-binding proteins in fatty acid uptake needs further evidence. Possibly they are necessary for uptake of ionized fatty acids and at low fatty acid concentrations for sequestering. They do not bind bilirubin, sulfobromophtalein, taurocholate, phosphatidylcholine or cholesterololeate.[338,339] Nothing more is known about their ligand specificity and their tertiary structure is completely unknown.

Transport of fatty acids across subcellular membranes has not been studied except for mitochondrial membranes. Activation and formation of acylcarnitine esters on the outer membrane are followed by translocation of the acylcarnitine through the inner membrane.[265] A specific translocase is involved in this latter step.[219]

The function of cytoplasmic FABP in fatty acid uptake was theoretically discussed for hepatocytes[239,355] and cardiomyocytes.[380] The fatty acid distribution between blood and cytoplasm may be, similar to that of retinol, dependent on the distribution between albumin and FABP. Intracellular levels of retinol are regulated by the level of CRBP and

retinol distribution between serum retinol-binding protein and CRBP is at equilibrium.[238] Evidence for the involvement of FABP in fatty acid uptake on basis of physiological data and results with inhibitors of fatty acid binding to FABP is equivocal,[374] but recently FABPs of Hep G2 cells, primary rat hepatocytes and differentiated 3T3 adipocytes were labeled in a time- and temperature-dependent fashion at the uptake of a photoactivable radioiodinated fatty acid analogue.[328,359,381,382] Transport of fatty acids from and to natural and model membranes has been observed for different types of FABPs.[63,163,164,209,253,330–332,397] The transfer function of FABP was also demonstrated in a model cytosol system.[326] The surface charge on the FABP and on the membrane of the cellular organelles may influence the transfer rate from FABP to membrane.[119,397]

With laser photobleaching the intracellular transport of a fluorescent fatty acid analogue was characterized in cultured hepatocytes.[192] The cytoplasmic diffusion rate and the fraction of cellular fluorescent fatty acid in aqueous cytoplasm was larger in female than male cells corresponding with their difference in FABP content.[23,152,240,248] The investigators suggested that FABP and other cellular binding proteins may enhance the diffusive flux of their ligand by reducing membrane binding.[192]

III. DISTRIBUTION AND STRUCTURE OF CYTOPLASMIC FATTY ACID-BINDING PROTEINS (FABPs)

A. *Distribution*

FABPs have been isolated from cytosols of tissues of vertebrates and invertebrates by various procedures, including gel filtration, ion-exchange and affinity chromatography, precipitation with salt or organic solvents, and preparative electrophoresis.[249] The purification methods had to be adapted for different tissues, not only due to different contaminating proteins. Tissue-specific FABP types appeared to exist, which were named after the first tissue of isolation. At the moment at least seven FABP types have been established on base of amino acid and/or cDNA sequence (Table 3). They show a characteristic tissue and cellular distribution. The presence of a certain FABP type is mostly established by isolation of the protein or by Western and/or Northern blots, but in some tissues the immunochemical or immunohistochemical analyses need additional evidence.

In some tissues a certain FABP type is limited to specific cell species, e.g. liver FABP to hepatocytes.[20,89,130,346,347] In other tissues more FABP types are present in different or similar cell types. Four FABP types are expressed in the stomach, depending on the cell type and the developmental stage.[4,129] The liver and heart FABP types are present in the kidney, but at different locations.[194,195] In intestine both the liver and intestinal FABP types are found in enterocytes of jejunum and ileum, but in colonocytes only the liver FABP

TABLE 3. Tissue Occurrence of FABP Types*

Liver type‡	Liver,† intestine,† kidney,† stomach
Intestinal type	Intestine,† stomach
Heart type	Heart,† kidney,† skeletal muscle,† aorta,† Adrenals,† placenta,† brain,† testes, ovary, Lung, mammary gland, stomach
Adipocyte type§	Adipose tissue†
Myelin type‖	Peripheral nervous system†
Ileal type¶	Intestine,† ovary, adrenals, stomach
Epidermal type	Skin†

*The indication of a FABP type in a tissue does not mean its presence in all cell types of that tissue; the FABP type may be limited to specific cells or may be present at certain developmental stages.

†Evidence was obtained both by protein and mRNA analysis.

‡Previously termed Z-protein or aminoazo dye-binding protein A or wrongly sterol carrier protein.

§Also named adipocyte lipid-binding protein or initially p422 or aP$_2$.

‖Originally named myelin P$_2$ protein.

¶Also named ileal lipid-binding protein and originally gastrotropin.

type.[68,281,287,306,347,349] The ileal type[95,151,287] is not only present in the ileum, but also in the ovary and adrenal gland and in surface mucous cells of the stomach of the rat.[4] The heart FABP type is the most general one (Table 3). Besides heart and skeletal muscle many other tissues contain this protein.[69,248,373,384] The intestinal, adipocyte,[26,31,207] myelin[118,223] and epidermal[198,308,309] FABP types are limited to one tissue or organ system. More information on the cellular and topographical distribution of the FABP types is given in the section on the FABP genes (Section IV).

Immunoelectron microscopy and analysis of isolated cell fractions indicated in most cases the presence of FABP in the cytoplasmic matrix outside the organelles.[249,374] Some investigators observed heart FABP in the mitochondria of rat heart[92] and bovine heart.[43,366] Liver FABP was never detected in mitochondria.[44,89,248] The ileal FABP type was detected in the nuclear matrix of ileal enterocytes.[4] Liver FABP was observed in the nucleus of bovine[44] and rat[89] hepatocytes and heart FABP in the nuclei of bovine heart[43] and locust flight muscle.[116]

B. *Primary Structure*

The family of FABPs and related proteins contains at least 11 members, the seven FABP types, CRBP I and II and CRABP I and II (Table 4). The proteins contain 126–137 amino acids and show 38–70% similarity of amino acid sequence.[374,375] The aligned amino acid sequences are given in Fig. 2. These alignments show the large similarity between the heart, myelin, adipocyte and epidermal FABP types and the marked gap in the C-terminal part of the liver FABP and ileal FABP type. Human heart and skeletal muscle FABP appeared to be identical proteins.[254] CRBPs and CRABPs show 20–45% similarity with the FABPs. The similarity is 56% between rat CRBP I and II[181] and 74% between mouse CRABP I and II[96] and between human CRABP I and CRABP II.[9] The FABP types differ considerably in their surface charge. The heart FABP type has an isoelectric point of about 5.0, the liver and epidermal FABP types of about 6.0 and the adipocyte and myelin FABP types are basic proteins (p*I* values of 7.6–9.0).

Table 4 gives some characteristics of the amino acid composition and sequence of the members of the FABP family. The absence of tryptophan and cysteine from liver and intestinal FABP type, respectively, are peculiar. The large similarity of heart, adipocyte, myelin and epidermal FABP types (60–70%) is reflected in the similar amino acids on essential positions for fatty acid binding (see IIIC). These latter FABP types and CRBP I and II contain a protein tyrosine kinase recognition sequence before Tyr[19]. The extent of phosphorylation of FABP is, however, very slight under various conditions *in vitro* and in 3T3-L1 adipocytes, rat cardiomyocytes and mammary epithelial cells.[30,50,51,124,231,232] The buried position of Tyr[19] in the FABP molecule does not allow kinase action and conformational changes would be necessary.[52,72] Phosphorylation of Tyr[19] seems, however,

TABLE 4. Characteristics of Members of the FABP Family

Member	Phe "17"	Phe "57"	Arg "106"	Arg "126"	Tyr "128"	Trp	Cys
Liver FABP	14	ser	thr	122	ser	—	68
Intestinal FABP	17	57	106	126	phe	6, 82	—
Heart FABP	16	55	106	126	128	8, 97	124
Adipocyte FABP	16	55	106	126	128	8, 97	117
Myelin FABP	16	55	106	126	128	8, 97	117, 124
Epidermal FABP	18	leu	108	128	130	10, 99	119, 126
Ileal FABP	17	gly	ala	123	ser	48	68
CRBP I	16	57	gln	gln	phe	8, 88, 106, 109	95, 126
CRBP II	16	57	gln	gln	phe	8, 88, 106, 109	121, 125
CRABP I	15	val	111	131	133	87, 109	95, 129
CRABP II	15	val	111	132	134	87, 109	95, 130

All differences are derived from human proteins, except for ileal FABP and CRBP II from pig and rat, respectively. Methionine on position 1 was not taken into consideration for numbering. Positions between quotation marks are compared with intestinal FABP and refer to amino acid residues involved in fatty acid binding or portal region.

```
          1
 L-FABP  MS....FSGK YQLQSQENFE AFMKAIGLPE EL..IQKGKD IKGVSEIVQN 43
 I-FABP  MA....FDST WKVDRSENYD KFMEKMGVNI VKRKLAAHDN LK..LTITQE 43
 H-FABP  MVDA..FLGT WKLVDSKNFD DYMKSLGVGF ATRQVASMT. .KPTTIIEKN 45
My-FABP  .SNK..FLGT WKLVSSENFD DYMKALGVGL ATRKLGNLA. .KPTVIISKK 45
 A-FABP  MCDA..FVGT WKLVSSENFD DYMKEVGVGF ATRKVAGMA. .KPNMIISVN 45
 E-FABP  MATVQQLEGR WRLVDSKGFD EYMKELGVGI ALRKMGAMA. .KPDCIITCD 47
Il-FABP  MA....FTGK YEIESEKNYD EFMKRLALPS DA..IDKARN LKIISEVKQD 43
  CRBPI  MPVD..FTGY WKMLVNENFE EYLRALDVNV ALRKIANLL. .KPDKEIVQD 45
 CRBPII  MTKD..QNGT WEMESNENFE GYMKALDIDF ATRKIAVRL. .TQTKIIVQD 45
 CRABPI  MPN...FAGT WKMRSSENFD ELLKALGVNA MLRKVAVAAA SKPHVEIRQD 46
CRABPII  MPN...FSGN WKIIRSENFE ELLKVLGVNV MLRKIAVAAA SKPAVEIKQE 46

 L-FABP  GKHFKFTITA GSKVIQNE.F TVGEECE..L ETMTGEKVKT VVQLEGDNKL 90
 I-FABP  GNKFTVKESS AFRNIEVV.F ELGVTFN..Y NLADGTELRG TWSLEGNKLI 90
 H-FABP  GDILTLKTHS TFKNTEIS.F KLGVEFDE.. TTADDRKVKS IVTLDGGKLV 92
My-FABP  GDIITIRTES TFKNTEIS.F KLGQEFEE.. TTADNRKTKS IVTLQRGSLN 92
 A-FABP  GDVITIKSES TFKNTEIS.F ILGQEFDE.. VTADDRKVKS TITLDGGVLV 92
 E-FABP  GKNLTIKTES TLKTTQFS.C TLGEKFEE.. TTADGRKTQT VCNFTDGALV 94
Il-FABP  GQNFTWSQQY PGGHSITNTF TIGKECD..I ETIGGKKFKA TVQMEG.GKV 90
  CRBPI  GDHMIIRTLS TFRNYIMD.F QVGKEFEEDL TGIDDRKCMT TVSWDGDKL. 93
 CRBPII  GDNFKTKTNS TFRNYDLD.F TVGVEFDEHT KGLDGRNVKT LVTWEGNTLV 94
 CRABPI  GDQFYIKTST TVRTTEIN.F KVGEGFEE.. ETVDGRKCRS LATWENENKI 93
CRABPII  GDTFYIKTST TVRTTEIN.F KVGEEFEE.. QTVDGRPCKS LVKWESENKM 93

 L-FABP  VTTFKNIKSV ......T.EL .NGDIITNTM TLGDIVFKRI SKRI. 126
 I-FABP  ...GKFKRTD NGNELNTVRE IIGDELVQTY VYEGVEAKRI FKKD. 131
 H-FABP  H....LQKWD GQETTLVREL I.DGKLILTL THGTAVCTRT YEKEA 132
My-FABP  Q....VQRWN GKETTIKRKL V.DGKMVAEC KMKGVVCTRI YEKV. 131
 A-FABP  H....VQKWD GKSTTIKRKR E.DDKLVVEC VMKGVTSTRV YERA. 131
 E-FABP  Q....HQEWD GKESTITRKL K.DGKLVVEC VMNNVTCTRI YEKVE 134
Il-FABP  VVNSPNYHH. ......TAEI V.DGKLVEVS TVGGVSYERV SKKLA 127
  CRBPI  ...QCVQKGE KEGRGWT.QW IEGDELHLEM RVEGVVCKQV FKKVQ 134
 CRBPII  C....VQKGE KENRGWK.QW VEGDKLYLEL TCGDQVCRQV FKKK  133
 CRABPI  HCTQTLLEGD GPKTYWTREL AND.ELILTF GADDVVCTRI YVRE. 136
CRABPII  VCEQKLLKGE GPKTSWTREL TNDGELILTM TADDVVCTRV YVRE. 137
```

Fig. 2. Alignment of the amino acid sequences of the members of the FABP family. All sequences are for human proteins, except ileal FABP and CRBP II, respectively for pig and rat. Identical residues present in at least 5 molecules are shaded.

to impede fatty acid binding to adipocyte FABP.[52] On the other hand, fatty acid binding activates phosphorylation of this protein.[124] Insulin receptor tyrosine kinase phosphorylated only less than 0.1% of human muscle FABP in *in vitro* experiments. (Prinsen and Veerkamp, unpublished data). Delipidation or fatty acid loading had no effect. No phosphorylation of FABP was observed with or without insulin stimulation in incubated rat soleus muscle or cultured rat muscle cells. The physiological relevance of tyrosine phosphorylation of FABP is therefore doubtful.

Heterogeneity of protein preparations (immunologically pure) has been found at chromatography and isoelectric focussing for various FABP types, especially for liver FABP.[208,352,374] Many possible causes have been suggested, as fatty acid loading, glutathione-FABP mixed disulfide formation, partial unfolding, N-acetylation, amidation of acidic residues. Recently it was established that amino acid exchange (Asp^{105}–Asn^{105}) and covalent modification by cysteine and glutathione could explain the heterogeneity of FABP from bovine liver.[81] The modifications did not affect fatty acid binding. Only one of two cytoplasmic isoforms of heart FABP was found in mitochondria of bovine heart.[366]

A high degree of similarity exists between the same FABP, CRBP or CRABP type from different mammalian species.[9,374] About the primary structure of members of FABP family in other vertebrates no data are available, although they were also isolated from various tissues of chicken and fish.[374] Muscle FABPs from two locusts, *Schistocerca gregaria*[116,263] and *Locusta migratoria*[193] show 41 and 42% identity of amino acid sequence with human muscle FABP[254] and a high percentage of conservative substitution, especially in residues involved in fatty acid binding.[193] The flatworm, *Schistosoma mansoni* contains a 15 kDa

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a predisposition to arteriosclerosis and in those who have hypertension resulting from an apparent underlying disease, such as pheochromocytoma. Called also *hyaline arteriolar n.* **hyaline arteriolar n.,** benign n. **hyperplastic arteriolar n.,** malignant n. **intercapillary n.,** arteriolar n. **malignant n.,** an uncommon form of arteriolar nephrosclerosis affecting all the vessels of the body, especially the small arteries and arterioles of the kidneys, and frequently associated with malignant hypertension and hyperplastic arteriolosclerosis. It may occur in the absence of previous history of hypertension, or may be superimposed on benign hypertension or primary renal disease, especially glomerulonephritis, benign nephrosclerosis, and pyelonephritis. Called also *hyperplastic arteriolar n.* and *Fahr-Volhard disease.* **senile n.,** nephrosclerosis which is just a part of the arteriosclerosis common in old age.

nephroscope (nef'ro-skōp) an instrument inserted into an incision in the renal pelvis for viewing the inside of the kidney, equipped with three channels for telescope, fiberoptic light input, and irrigation.

nephroscopy (nĕ-fros'ko-pe) visualization of the kidney by means of the nephroscope.

nephroses (nĕ-fro'sēz) plural of *nephrosis.*

nephrosis (nĕ-fro'sis), pl. *nephro'ses* [*nephr-* + *-osis*] any disease of the kidney, especially any disease of the kidneys characterized by purely degenerative lesions of the renal tubules—as opposed to nephritis—and marked by edema (noninflammatory), albuminuria, and decreased serum albumin (the nephrotic syndrome). **acute n.,** nephrosis marked by scanty urine but with little edema or albuminuria. **amyloid n.,** chronic nephrosis with amyloid degeneration of the median coat of the arteries and the glomerular capillaries; amyloid kidney. **avian n.,** infectious bursal disease. **cholemic n.,** renal disease associated with various types of hepatic or biliary dysfunction, especially those in which there is obstructive jaundice. **chronic n.,** renal disease characterized by chronic degeneration of the renal epithelium. **Epstein's n.,** a type of chronic tubular nephritis resulting from systemic metabolic disorder, occurring usually in young persons and in women, and frequently associated with hypothyroidism or other endocrine disturbance. **glycogen n.,** nephrosis associated with glycogen vacuolation within the proximal convoluted tubules and the loops of Henle. **hydropic n.,** vacuolar n. **hypokalemic n.,** vacuolar n. **infectious avian n.,** infectious bursal disease. **larval n.,** a condition in which the renal lesions are slight and manifested clinically by albuminuria. **lipid n., lipoid n.,** nephrosis characterized by edema, albuminuria, and changes in the protein and lipids of the blood and the accumulation of globules of cholesterol esters in the tubular epithelium of the kidney. **lower nephron n.,** a condition of renal insufficiency leading to uremia, due to necrosis of the cells of the lower nephron, blocking the tubular lumens of this region. The condition is seen after severe injuries, especially crushing injury to muscles (*crush syndrome*). **necrotizing n.,** renal disease characterized by necrosis of tubular epithelium of the kidney. **osmotic n.,** vacuolar n. **toxic n.,** nephrosis caused by some toxic agent, most frequently and typically by bichloride of mercury. **vacuolar n.,** renal disease in which injury of the renal tubules is associated with vacuolization of the proximal convoluted tubules and sometimes of the loops of Henle and collecting tubules, presumed to be caused by disturbances in the normal osmotic relationships within the cells. These changes are seen in various clinical situations, as following the administration of hypertonic solutions, in diseases resulting in marked alterations in fluid balance, and in severe hypokalemia. Called also *hydropic n., hypokalemic n.,* and *osmotic n.*

nephrosonephritis (nĕ-fro"so-nĕ-fri'tis) [*nephrosis* + *nephritis*] renal disease with nephrotic and nephritic components. **hemorrhagic n., Korean hemorrhagic n.,** epidemic hemorrhagic fever.

nephrosonography (nef"ro-so-nog'rah-fe) ultrasonic scanning of the kidney.

nephrospasis (nef"ro-spas'is) [*nephro-* + Gr. *span* to draw] movable kidney in which the natural supports of the organ are so weakened that the organ hangs by its pedicle.

nephrostolithotomy (nĕ"fro-sto-lĭ-thot'o-me) [*nephro-* + Gr. *lithos* stone + Gr. *tomē* a cutting] removal of renal calculi through a nephrostomy tube inserted through the abdominal wall into the renal pelvis.

nephrostoma (nĕ-fros'to-mah) [*nephro-* + Gr. *stoma* mouth] one of the funnel-shaped and ciliated orifices of excretory tubules that open into the coelom in the embryo, best seen in lower vertebrates.

nephrostome (nef'ro-stōm) nephrostoma.

nephrostomy (nĕ-fros'to-me) [*nephro-* + Gr. *stomoun* to provide with an opening, or mouth] the creation of a fistula leading directly into the pelvis of the kidney.

nephrotic (nĕ-frot'ik) pertaining to, resembling, or caused by nephrosis.

nephrotome (nef'ro-tōm) one of the segmented divisions of the mesoderm connecting the somite with the lateral plates of unsegmented mesoderm; it is the source of much of the urogenital system. Called also *intermediate cell mass* and *middle plate.*

nephrotomogram (nef"ro-to'mo-gram) the sectional radiograph of the kidney obtained by nephrotomography.

nephrotomography (nef"ro-to-mog'rah-fe) radiologic visualization of the kidney by tomography after intravenous introduction of contrast medium as a bolus or by infusion.

nephrotomy (nĕ-frot'o-me) [*nephro-* + Gr. *tomē* a cutting] a surgical incision into the kidney. **abdominal n.,** nephrotomy performed through an incision into the abdomen. **anatrophic n.,** incision into the kidney between its vascular segments, to minimize bleeding and parenchymal injury and to prevent atrophy. **lumbar n.,** nephrotomy performed through an incision into the loin.

nephrotoxic (nef"ro-tok'sik) toxic or destructive to kidney cells.

nephrotoxicity (nef"ro-tok-sis'ĭ-te) the quality of being toxic or destructive to kidney cells.

nephrotoxin (nef"ro-tok'sin) [*nephro-* + Gr. *toxikon* poison] a toxin which has a specific destructive effect on kidney cells.

nephrotropic (nef"ro-trop'ik) having a special affinity for or exerting its principal effect upon kidney tissue.

nephrotuberculosis (nef"to-tu-ber"ku-lo'sis) [*nephro-* + *tuberculosis*] disease of the kidney due to *Mycobacterium tuberculosis.*

nephroureterectomy (nef"ro-u"rĕ-ter-ek'to-me) [*nephro-* + *ureterectomy*] excision of a kidney and a whole or part of the ureter.

nephroureterocystectomy (nef"ro-u-re"ter-o-sis-tek'to-me) [*nephro-* + Gr. *ourētēr* ureter + *kystis* bladder + *ektomē* excision] excision of the kidney, ureter, and a portion of the bladder wall.

nephrozymosis (nef"ro-zi-mo'sis) zymotic or fermentative disease of the kidney.

nephrydrosis (nef"rĭ-dro'sis) [*nephro-* + Gr. *hydōr* water + *-osis*] hydronephrosis.

nephrydrotic (nef"rĭ-drot'ik) pertaining to nephrydrosis.

nepiology (nep"e-ol'o-je) [Gr. *nēpio* infant + *-logy*] (*obs.*) the department of pediatrics treating of young infants.

Neptazane (nep'tah-zān) trademark for a preparation of methazolamine.

neptunium (nep-tu'ne-um) [from planet Neptune] a radioactive element of atomic number 93 and atomic weight 237, occurring in certain earths and obtained by splitting the uranium atom with neutrons. It is unstable and changes into plutonium. Symbol Np.

nequinate (nĕ-kwin'āt) chemical name: 6-butyl-1,4-dihydro-4-oxo-7-(phenylmethoxy)-3-quinolinecarboxylic acid methyl ester; a coccidiostat for poultry, $C_{22}H_{23}NO_4$.

Nerium (ne'rĭ-um) a genus of evergreen apocynaceous shrubs of the Mediterranean region and Asia. *N. odorum* (*N. indicum*) is the sweet-scented oleander; *N. oleander* L. is the common oleander.

Nernst equation, potential (nernst) [Walther Hermann *Nernst,* German physical chemist, 1864–1941] see under *equation* and *potential.*

nerol (ne'rol) an essential oil, $(CH_3)_2C{:}CH \cdot CH_2 \cdot CH_2 \cdot C(CH_3){:}CH \cdot CH_2OH$, a constituent of orange flower oil.

neroli (ner'o-le) an essential oil distilled from orange blossoms; orange flower oil.

nerval (ner'val) (*obs.*) neural.

nerve (nerv) [L. *nervus;* Gr. *neuron*] a cordlike structure,

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IN THE U.S. PATENT AND TRADEMARK OFFICE

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Appl. No.:	09/578,693	Group:	1641
Filed:	May 26, 2000	Examiner:	L. Cook
For:	METHOD FOR EXAMINING HUMAN KIDNEY DISEASE BY DETECTING THE FATTY ACID BINDING PROTEIN		

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Takeshi SUGAYA, a citizen of Japan residing at Itami-shi, Hyogo-ken, Japan, hereby declare as follows:

1. I am a co-inventor of the subject matter of the above-referenced patent application.

2. I graduated from the Faculty of Engineering of Kyoto University, Japan in March 1986. I also received my Masters degree in Engineering from Kyoto University in March 1989 and my Ph.D from the University of Tsukuba in November 1999.

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above-identified application. From April 1989 until October 2000 I was engaged in research works in the fields of biochemistry and pharmacology at research laboratories of Tanabe Seiyaku Co., Ltd. From November 2000 to present, I have been on loan to CMIC Co., Ltd., Tokyo, Japan, an outsourcing pharmaceutical development company, one of the Contract Research Organizations (CROs). At CMIC Co., Ltd., I have been engaged in research and development of diagnostic reagents, and methods and systems including the same in the field of diagnosis of renal disease directed by L-FABP. My present position is as the project leader of business development at CMIC Co., Ltd.

4. I am the co-author of about 53 papers in the field of cardiovascular and nephrology, and a co-author of 5 papers (one being still in press) in the field of diagnosis of renal disease focused on L-FABP. I received the young investigator award from the Society of Japan Cardiovascular Endocrinology and Metabolism in 1997. Since January 2005 to present, I was delegated as a guest professor at the department of Nephrology and Hypertension at St. Marianna University School of Medicine, Japan.

5. I have read and understood the contents of U.S. patent application no.: 09/578,693 and am familiar with the prosecution history of said application.

6. The foregoing remarks and objective evidence are presented to show that the claimed invention satisfies a long-felt need in the art, which was recognized, persistent, and not solved by others until conception and reduction to practice of the presently claimed invention. For this reason, the claimed invention is novel and non-obvious over the prior art.

7. The need for a method for the diagnosis or prognosis of kidney disease in a human is a persistent one that is recognized by those of ordinary skill in the art. The American Heart Association Science Advisory and Coordinating Committee states that kidney disease is a worldwide public health problem. *See*, Sarnak et al., "AHA Scientific Statement: Kidney disease as a risk factor for development of cardiovascular disease," *Circulation*, 108:2154-2169, October 28, 2003 (attached hereto as **Exhibit A**). As described in the Scientific Statement, chronic kidney disease is a major health problem facing the world today, and the care and treatment of

patients suffering from kidney disease imposes a terrible economic burden on society.

In the United States, for example, the number of individuals with kidney failure treated with dialysis and kidney transplantation exceeded 320,000 in 1998, and continues to increase. At the present time, the prevalence of patients in the early stages of chronic kidney disease is even greater. The Scientific Statement also states that cardiovascular disease (CVD) is frequently associated with kidney disease, and that patients with kidney disease associated with CVD tend to die of CVD. Thus, the diagnosis/prognosis of kidney disease is also very important as viewed as a risk factor for CVD. *See*, AHA Scientific Statement, page 2154, left column.

Since kidney disease has become and is a major worldwide public health problem, a method for the diagnosis/prognosis of kidney disease has taken on a great importance in society. However, prior to the present invention, no sufficient technology for the diagnosis/prognosis of kidney disease has been developed. For example, as described in Levin, "Consequences of late referral on patient outcomes," *Nephrol. Dial. Transplant*, 15(Suppl.3):8-13, 2000 (attached hereto as **Exhibit B**), the identification of patients in the early stages of chronic kidney disease is critical to good patient outcome. Levin notes, however, that under the current state of the art,

most patients are referred to nephrologists too late in the course of their renal disease for improvement. As a consequence of these late referrals to nephrologists, there is a significant increase in patient morbidity, mortality and resource utilization.

Such dire consequences would be avoidable if an adequate method for the diagnosis/prognosis of kidney disease existed. In this regard, Levin expressly blames the insensitivity of current screening tools for causing late referrals: "Reasons for late referral include **insensitivity of current screening tools**. Serum creatinine is well known to be an **inaccurate marker of renal dysfunction**, and too insensitive to identify patients with very early stages of disease, thus contributing to the prevalence of late referrals (emphasis added)." *See*, page 8, left column, 4th paragraph. See also, page 10, right column, 1st paragraph, where Levin states: "However, current patterns of 'late referrals' may reflect, at least in part, **difficulties with today's screening tools** (emphasis added)."

Again, the consequences of these late referrals are dire: "The consequences of late referrals include **increased morbidity, mortality and resource utilization**. There is also an impact on patients' quality of life and **missed opportunities** for pre-emptive transplantation (emphasis added)." *See*, page 8, right column, 2nd paragraph. As such,

the art recognizes that there is a direct correlation between the current deficiencies in the art and poor outcome for patients suffering from kidney disease.

Levin goes on to expressly state that poor patient outcome caused by late referral could be avoided if there were an adequate method for diagnosing kidney disease: "The first step in ensuring timely referral of patients to nephrologists is the implementation of sensitive screening tools (emphasis added)." *See*, page 10, left column, 2nd paragraph, last 3 lines.

Given the above statements, Levin recognizes the persistent need that exists in the art for a method for the diagnosis or prognosis of kidney disease in a human. Levin further evidences that this need was well recognized by those of ordinary skill in the art. Thus, the current development of a more practical screening tool (i.e., method for diagnosis/prognosis of kidney disease) represents a significant advance in the art.

8. The long-felt need in the art for a method for the diagnosis or prognosis of kidney disease in a human was not satisfied by another before the presently claimed invention was invented by my co-inventors and I.

The diagnostic techniques currently employed to screen for kidney disease all have various defects as described in, for example, Caramori et al., "Perspectives in Diabetes: The need for early predictors of diabetic nephropathy risk. Is albumin excretion rate sufficient?" *Diabetes*, 49:1399-1408, September 2000 (attached hereto as **Exhibit C**), and Rodrigo et al., "Measurement of renal function in pre-ESRD [end-stage renal disease] patients" *Kidney International*, 61(Suppl.80):S11-S17, 2002 (attached hereto as **Exhibit D**).

Specifically, Caramori et al. evidences that the current method of measuring albumin excretion rate, which is currently the best available noninvasive predictor of diabetic nephropathy (DN) risk, is still insufficient under many circumstances:

> However, AFR (albumin excretion rate) may be unable to define patients who are safe from or at risk of DN with an accuracy that is adequate for optimal clinical decision making or for the design of certain clinical trials. Investigations into new risk markers or into the combined use of several currently available predictive parameters are needed.

See, page 1399, left column, line 3 from the bottom to right column, line 4.

In addition, Rodrigo et al. evidences that the most reliable methods currently used in the art are deficient:

> The most reliable methods, such as inulin clearance or measurement by radioisotopes, are too awkward for the usual clinical follow-up of patients...The determinations of the plasmatic creatinine and its clearance or the estimate of the glomerular filtration rate by means of equations derived from the creatinine are the methods most often used in order to measure renal function, although not without problems in pre-dialysis. In order to try to overcome such problems, more precise equations and procedures, including the measurement of averaged urea-creatinine clearance or creatinine clearance with cimetidine, have been designed that better estimate the glomerular filtration rate. **However, none of these methods is totally reliable in pre-dialysis** (emphasis added).

See, page S11, left column.

Since the diagnosis/prognosis of kidney disease is very important for determining the most suitable method for treatment, a new method for the diagnosis/prognosis has earnestly been desired in the art. Nevertheless, no satisfactory method for the diagnosis or prognosis of kidney disease has ever been disclosed prior to the filing of the present patent application. In fact, the present invention can be used not only for diagnosis but also for the prognosis of kidney disease, which hitherto has been very difficult. Hence, the present invention is extremely effective and useful in a practical standpoint.

9. The present invention in fact satisfies the long-felt need in the art for a method for diagnosis/prognosis of kidney disease in a human.

The claimed method of the present invention is vastly superior over the existing methods. The effectiveness and usefulness of the present invention are clear from the data published in Kamijo et al., *J. Lab. Clin.* Med. 143(1):23-30, 2004 (already of record in the present application).

In Kamijo et al., the correlation of diagnostic markers, including L-FABP of the method of the present invention, with the progression rate of renal disease was statistically analyzed in patients with chronic kidney disease. The clinical test results are shown in Table III and page 26, left column, last line to the right column, line 8. The "F ratio" in Table III stands for the correlation with "progression rate." This means that the larger the F ratio, the higher the correlation. As is clear from the test results, among the various diagnostic markers (e.g., serum creatinine, urinary protein, NAG, α1-MG, etc.), **only L-FABP had a statistically significant high F ratio (F ratio = 17.1)**. On the other hand, all of the other diagnostic markers only had an F ratio of between 0.1 and 2.0. These results unequivocally prove that L-FABP has a very high correlation with the progression rate

of renal disease.

In the abstract, Kamijo et al. states, "The results showed that urinary L-FABP reflected the clinical prognosis of chronic renal disease. Urinary L-FABP may be a clinical marker that can help predict the progression of chronic glomerular disease." See, page 23, abstract, lines 19-22. Thus, it has been confirmed that the method of the present invention is superior and can also be applied to prognosis of kidney disease.

As is further noted in Kamijo et al., the method of the present invention has been subjected to clinical tests and applications for the development of new diagnostic reagents in Japan and now plans are in place for clinical tests also in the United States.

When the present invention is used clinically, it will highly contribute to the remedy of the kidney disease and then to resolution of the social problems due to the kidney disease as mentioned above.

10. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or

imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Signed this day 10 of May, 2005

Takeshi Sugaya

Takeshi SUGAYA

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant:	YAMANOUCHI, Masaya et al.	Conf:
Appl. No.:	09/578,693	Group: 1641
Filed:	May 26, 2000	Examiner: L. Cook
For:	METHOD FOR EXAMINING HUMAN KIDNEY DISEASE BY DETECTING THE FATTY ACID BINDING PROTEIN	

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

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6. Under my direction, the following experiment has been done.

[I] Object of the experiment:

The following experiment has been done in order to prove that the method of the present invention in which L-FABP is detected as a marker show superior effectiveness over a method in which H-FABP is detected, and to prove that L-FABP and H-FABP are not similar and/or equivalent, and H-FABP is not an obvious substitute for L-FABP.

[II] Methods:

(1) Subjects:

Urine samples were collected from seven human individuals having moderate kidney dysfunction and who underwent radiographic examination procedures (coronary angiography) accompanied by administration of contrast media.

The individuals were those with serum creatinine level greater than normal range(*) [5 men and 2 women; mean age, 66 years old; mean value of the serum creatinine level were 1.31mg/dL in men and 1.05mg/dL in women]. [*: upper limit of normal range in the hospital was 1.04 mg/dL in men and 0.79 mg/dL in women].
In the study, individuals with the following were not included: end-stage renal disease under hemodialysis, liver disease and cancer.

The contrast media used was a non-ionic low-osmolarity type agent.

Before and 8, 16, 24 and 48 hours after administration of the contrast media, the urine samples were collected from each individuals, and the level of FABPs (L-FABP

and H-FABP) in the urine samples were measured. The concentration of FABPs in the samples was measured as described in the following (2) and (3).
The concentration of creatinine in the samples was measured by conventional enzymatic method. The value of FABP concentration was corrected by the value of urinary creatinine concentration to be shown as the level of urinary FABP (μg/ g creatinine).

(2) L-FABP assay:

L-FABP in urine sample (urinary L-FABP) was measured by sandwich ELISA (Enzyme-linked immunoabsorbent assay).
The assay was carried out by using a kit containing monoclonal antibodies specific to human L-FABP (human L-FABP ELISA kit; CMIC Co., Ltd.(Japan)), and following the detailed instructions described in the manual of the kit.

Briefly, samples treated with pretreatment solution were transferred into anti-L-FABP antibody immobilized microplate containing assay diluent.
After incubation, the microplate was washed and added with a reagent containing peroxidase-conjugated secondary antibody. After incubation, the microplate was washed and added with substrate for enzyme reaction. After reaction, the optical density was measured using a microplate reader, and calibration curve was prepared based on the obtained optical density, thereby determining the L-FABP concentration.

(3) H-FABP assay:

H-FABP in urine sample (urinary H-FABP) was also measured by sandwich ELISA as in the above (2). The assay was carried out by using a kit containing monoclonal antibodies specific to human H-FABP (human H-FABP ELISA kit; HyCult biotechnology b.v. (Netherlands)), and following the detailed instructions described in the manual of the kit.

Breifly, samples were transferred into anti-H-FABP antibody-immobilized microplate containing assay diluent. Thereto were added a reagent containing peroxidase-conjugated secondary antibody. After incubation, the microplate was washed and added with substrate for enzyme reaction. After reaction, the optical density was measured using a microplate reader, and calibration curve was prepared based on the obtained optical density, thereby determining the H-FABP concentration.

[III] Results:

It is known that administration of contrast media used in radiographic examination procedures is a load/stress for kidney and cause induction of renal injury.

In this study, before and after administration of the contrastmedia to individuals who underwent radiographic examination procedure, the urine samples were collected and the level of L-FABP were measured to study the change with lapse of time to monitor the renal injury induced by administration of contrast media.

In addition, as a comparative study, the level of H-FABP in the same samples were measured.

The results are shown in Table 1, and the same is graphed out in Figure 1.

As shown in Table 1 and Figure 1, the level of urinary L-FABP has distinctly elevated with lapse of time after administration of contrast media. However, such was not observed in the level of urinary H-FABP. The level of urinary H-FABP hardly changed and was at a low level throughout the course of time.

The results of this study show that the renal injury induced by contrast media can be sensitively examined by measuring the level of L-FABP, however, it cannot be done as well by measuring H-FABP instead of L-FABP.

Table 1

	Urinary FABP (μg/g creatinine) Mean±S.D.				
	Pre*	8 hr **	16 hr **	24 hr **	48 hr **
L-FABP	4.24±2.98	11.58±12.95	30.12±23.02	44.68±18.62	26.64±25.45
H-FABP	0.08±0.20	0.71±0.94	0.73±1.16	1.17±1.48	0.11±0.29

*: before administration of contrast media
**: time after administration of contrast media

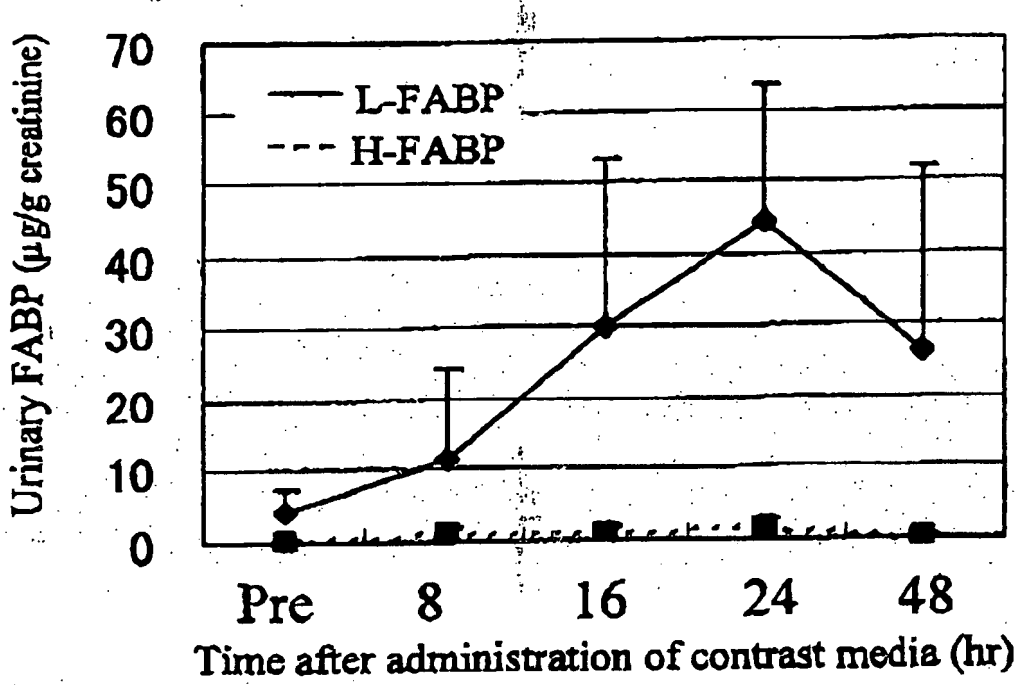

Fig. 1

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Signed this day 14 of March, 2006

Takeshi SUGAYA

Original Article *Ann Clin Biochem* 1997; 34: 263–268

One-step enzyme-linked immunosorbent assay (ELISA) for plasma fatty acid-binding protein

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From the [1]Department of Physiology, Cardiovascular Research Institute Maastricht, University of Limburg, PO Box 616, NL-6200 MD Maastricht, [2]Department of Clinical Chemistry, Academic Hospital Maastricht, Maastricht, The Netherlands, and [3]Roche Diagnostic Systems, a division of F. Hoffman-La Roche Ltd, Basel, Switzerland

SUMMARY. To allow a more rapid determination of heart-type fatty acid-binding protein (FABP) concentration in plasma a direct non-competitive (sandwich-type) ELISA was developed which uses high-affinity monoclonal antibodies to FABP. Total performance time of the one-step immunoassay is 45 min. The standard curve was linear between 0·2–6 μg/L, and the within-run and between-run coefficients of variations were below 6 and 11%, respectively. The serum FABP concentration measured in 79 healthy individuals was 1·6 (0·8) [mean (SD), range 0·3–5·0] μg/L. The assay can be used for rapid plasma or serum FABP measurement in the early diagnosis of acute myocardial infarction.

Additional key phrases: heart-type fatty acid-binding protein; monoclonal antibodies

Fatty acid-binding protein (FABP) is a recently introduced plasma marker of acute myocardial infarction (AMI) in man.[1-4] The plasma kinetics of FABP (15 kDa) closely resemble those of myoglobin (18 kDa) in that significantly elevated plasma concentrations are found within 3 h after AMI which generally return to normal values within 12 to 24 h.[5-7] These features make FABP a useful biochemical marker especially for the early assessment or exclusion of AMI,[5,6] and for the monitoring of a recurrent infarction.[7] Since FABP released from the heart after AMI is quantitatively recovered in plasma, FABP can also be used to estimate infarct size.[8]

As with myoglobin, small quantities of (heart-type) FABP are also found in skeletal muscle, which are released into the circulation following injury.[7] However, as the ratio of the tissue concentrations of myoglobin over FABP is different in heart (4–5) and skeletal muscles (20–70), the assessment of this ratio in plasma allows the discrimination between myocardial and skeletal muscle injury.[7]

The clinical application of FABP to confirm or exclude a diagnosis of AMI soon after admission requires a rapid test system, which is not yet available. Because FABP does not exhibit enzymatic activity, its plasma concentration has to be measured immunochemically. However, reported immunochemical assays for FABP take about 2 to 5 h to complete.[2,3,5,9] Here we describe a one-step sandwich-ELISA for FABP in plasma which uses high-affinity monoclonal antibodies, and shows a high sensitivity with a total performance time of only 45 min.

Correspondence: Jan F C Glatz PhD.

MATERIALS AND METHODS

Collection of blood samples

For determination of the reference range of FABP in serum and plasma, blood samples were withdrawn from 79 healthy subjects (61 men, median age 35, range 20–51 years; and 18 women, median age 38, range 23–51 years). Subjects were taking no medication. Blood samples were obtained between 9 and 12 am and collected in glass tubes (preparation of serum). For 10 subjects, a second blood sample was collected in glass tubes containing dry heparin (preparation of plasma). After centrifugation at 1500*g* for 10 min, serum or plasma was collected and stored at −70°C until analysis.

Comparison of the present assay with a previously described immunochemical assay for FABP was carried out on serial plasma samples from patients with confirmed AMI. We studied six randomly selected patients (five men, one woman, median age 58, range 52–61 years) from a population of 22 patients enrolled in a recent study.[10] Patients were admitted to the coronary care unit with chest pain and ST segment elevation (> 1 mm) typical of AMI within 6 h after the onset of symptoms. They received thrombolytic therapy consisting of 1·5 million units of streptokinase given by infusion in 40 min. Blood samples, obtained at admission and subsequently at nine time points (final sample was taken 24 h after admission), were collected in dry heparin-containing tubes and processed exactly as described above.

Isolation and purification of FABP

Human heart-type FABP, used as standard in the calibration curves, was purified from human heart autopsies by gel permeation and anion-exchange chromatography as described previously.[5] In addition, we used recombinant human FABP, kindly donated by Dr T Börchers (University of Münster, Germany).

Monoclonal-antibody based assay of FABP

For measurement of FABP in serum or plasma, a direct non-competitive ELISA of the antigen capture type (sandwich ELISA) was developed, based on the use of monoclonal antibodies (mAb). Thirteen mAbs, all of subtype IgG1 and directed against purified human heart-type FABP, were raised by the classical hybridoma technology and characterized by surface plasmon resonance analysis using a Pharmacia BIAcore biosensor, as described in detail elsewhere.[11] The mAbs recognized five distinct (three independent and two overlapping) epitopes on human FABP, and showed no cross-reactivity with human intestinal-type and human liver-type FABP. Seven of the mAbs were selected on the basis of stability of the hybridoma clone and affinity for FABP. Affinity constants for FABP interactions with these selected mAbs ranged from 5×10^7 to $3 \times 10^8 M^{-1}$.[11] Antibodies were stored in buffer solution at −20°C, and conjugated antibodies at 4°C, and were found to be stable for more than 12 months.

Assays were carried out on polyvinylchloride microtitre plates (Falcon type 3912; Becton Dickinson, Oxnard, CA, USA). Plates were coated overnight at 4°C with 200 ng per well of a specific mAb in 100 μL 0·1 M sodium bicarbonate buffer (pH 9·4). The wells then were washed five times with phosphate-buffered saline containing 0·1% (w/v) bovine serum albumin (A4503 from Sigma, St Louis, MO, USA) and 0·05% (v/v) Tween-20 (PBT). Thereafter, 50 μL of a solution containing 180 ng/mL of mAb conjugated with horseradish peroxidase (HRP; P8375 from Sigma, St Louis, MO, USA) and recognizing a different epitope group on FABP was added to each well. This was immediately followed by 50 μL of the standard containing 0–12 μg/L FABP (0–600 pg purified human FABP per well), or 50 μL of 25-fold prediluted human serum or plasma samples (fivefold predilution in case of healthy subjects). After incubation for 30 min at 37°C the microtitre plates were washed five times with PBT and developed with 100 μL per well of a substrate solution consisting of 20 mmol/L tetramethyl benzidine (TMB) and 6 mmol/L H_2O_2 dissolved in 0·1 M sodium citrate buffer (pH 5·0). After 5 min, the reaction was stopped with 50 μL of 2 M H_2SO_4 per well, and the absorbance was read at 450 nm using a Titertek Multiscan MKII microplate reader. The detection limit of the assay was 0·2 μg/L (20 pg/well).

Polyclonal-antibody based assay of FABP

The newly developed mAb based sandwich ELISA was compared with the polyclonal-antibody (pAb) based ELISA described previously.[5] The latter assay was carried out in a similar manner as outlined above, but used monospecific polyclonal IgG antibodies isolated by affinity chromatography from rabbit serum. After coating the wells with capture antibody, blocking with 5% (w/v) bovine serum albumin, and addition of standards or diluted plasma samples, incubation took place for 1·5 h at room temperature. The plates then were washed five times with PBT and incubated for another 1·5 h with 60 ng/well biotinylated monospecific polyclonal rabbit IgG (detector antibody) in 100 μL of PBT. Thereafter, the plates were washed again and detection of the biotinylated antibody bound to FABP was achieved by incubation with 100 μL of streptavidine-HRP (Pierce, Rockford, IL, USA) for 1 h, followed by washing and development with 100 μL per well of a substrate solution consisting of 20 mmol/L O-phenylene diamine (OPD) and 6 mmol/L H_2O_2 in 0·1 M sodium citrate buffer (pH 5·0). After 5 min, the reaction was stopped with 50 μL

of 2 M H_2SO_4 and the absorbance was read at 492 nm. Detection limit of the assay was 1 μg/L (50 pg/well).[5]

Statistical analysis

Data are presented as mean (SD). Statistical analysis of differences between groups was performed with Student's *t*-test. The level of significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Performance of monoclonal antibody-based assay

The seven different mAbs selected on the basis of stability of the hybridoma clone and affinity for FABP were tested both as capture and as detector antibodies (the latter conjugated with HRP) in different combinations by a checkerboard approach. For combinations of mAbs directed against distinct epitope groups a similar linear FABP standard curve was found, with the absorbance at a specific FABP concentration differing maximally twofold from that observed with the eventually selected mAbs. Optimal results were obtained with the combination of mAb 67D3 immobilized on the polyvinylchloride microtitre wells (capture antibody) and mAb 66E2 conjugated with HRP as detector antibody. The standard curve was found to be linear up to 6 μg/L (600 pg/well) FABP, and no differences were found between a standard curve recorded with FABP isolated from human heart and that recorded with recombinant human FABP (the absorbance with the latter was 97±5% ($n=3$) (Fig. 1). The linearity of the standard curve would allow reducing the number of standards to two or even one, thus facilitating the use of this assay for patient samples. Reduction of the incubation time from 30 to 15 min did not affect the linearity of the standard curve, but increased the intra-assay variability to above 10% so that the routine incubation time was set at 30 min (data not shown).

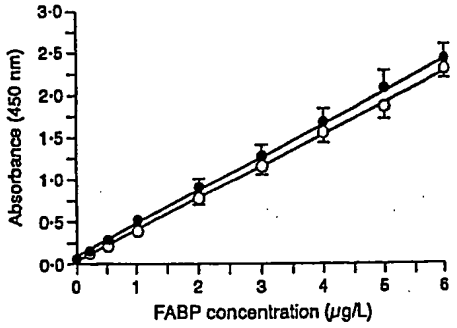

FIGURE 1. *Standard curves for the one-step sandwich enzyme-linked immunoasborbent assay (ELISA) for heart-type fatty acid-binding protein (FABP), using monoclonal antibodies and tissue-derived (●) or recombinant (○) human heart FABP. Data refer to mean (SD) for 10 curves. For the lower values the SD is not larger than the size of the symbols used.*

Assay variation and linearity of dilution

To estimate the imprecision of the assay two different human plasma pools with elevated FABP concentrations were analysed three times each on 10 consecutive days. For the pooled plasma sample with a relatively low FABP concentration (26 μg/L) the calculated intra-assay coefficient of variation (CV) was 6%, and the inter-assay CV 11%; for the pooled plasma sample with a high FABP concentration (280 μg/L) the intra-assay CV was 5% and the inter-assay CV 9%.

The linearity of dilution was investigated using the plasma samples with a high FABP concentration. Serial dilutions (up to 10-fold) were made in saline. After correction of the measured value for the dilution factor, recovery was calculated to be between 97 and 113%. Recovery of pure human FABP added in various quantities to plasma of healthy individuals amounted to 97 (5)% [mean (SD) for 12 determinations].

Method comparison

Fifty-four plasma samples with FABP concentrations between 2 and 310 μg/L, as assessed with the existing immunochemical assay based on polyclonal antibodies, were re-evaluated using the newly developed mAb ELISA. The correlation found was $y=1.04x+0.23$ ($r=0.99$). Agreement between the two methods was analysed in a difference plot according to Bland and Altman (Fig. 2).[12] The accuracy was assessed by calculating the 95% confidence interval [mean (2 SEM)] for the mean of the differences (4·3 μg/L). As this interval (−0·8–7·8 μg/L) includes zero, there is no evidence of systematic bias. With respect to the precision, the scatter of the differences was found to increase as the average increased (Fig. 2), but the limits of agreement [mean (2 SD) i.e. −21·5 and +30·2 μg/L] are acceptably small. We conclude that there is agreement between the methods.

To determine the stability of FABP in plasma samples on storage and following repeated cycles of freezing and thawing, six plasma

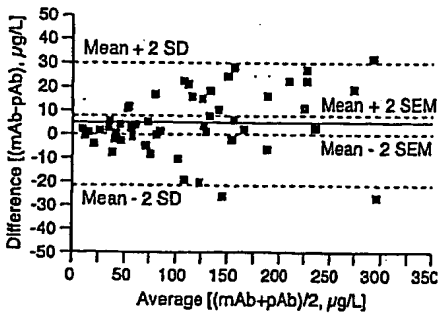

FIGURE 2. *Difference plot of 54 plasma samples in which FABP concentration was measured by monoclonal antibody-based (mAb) and polyclonal antibody-based (pAb) immunochemical assay. Mean difference (solid line), 95% confidence interval for the mean difference [mean (2 SEM) dashed line], and limits of agreement [mean (2 SD) dashed line] are shown.*

samples with FABP concentrations ranging from 25 to 250 µg/L were assayed freshly, then divided into aliquots and stored at 4°C for 1 week, and at −20°C and −70°C for 12 months. When compared to fresh plasma samples, no significant alterations in FABP concentration were found in these samples as analysed in the given time periods. Repeated freezing and thawing (tested up to eight times) also had no influence on measured FABP concentrations (data not shown). The high stability of FABP in plasma agrees with observations made by Ohkaru *et al.*[9]

Reference values

The mAb ELISA was used to measure the FABP concentration in serum and heparinized plasma samples from 79 apparently healthy individuals (Table 1). Men showed a slightly higher concentration of FABP [1·6 (0·8) µg/L, mean (SD) for $n=61$) than women [1·4 (0·5) µg/L, mean (SD) for $n=18$), but the difference was not significant. FABP concentrations measured in serum did not differ from those measured in heparinated plasma [plasma/serum ratio 1·04 (0·08) mean (SD), $n=10$].

TABLE 1. *Fatty acid-binding protein (FABP) concentration in serum samples from healthy subjects*

Sex	Age	n	FABP concentration (µg/L) Mean (SD)	Range
Women	20–30	5	1·0 (0·6)	0·3–1·7
	31–40	8	1·6* (0·4)	1·0–2·2
	41–51	5	1·6 (0·6)	0·7–2·4
	All	18	1·4 (0·5)	0·3–2·4
Men	20–30	19	1·2 (0·4)	0·5–2·0
	31–40	30	1·8* (0·9)	0·9–5·0
	41–51	12	1·8* (0·9)	0·9–3·7
	All	61	1·6 (0·8)	0·5–5·0

*Significantly different from value at age 20–30 ($P<0·05$).

Serum FABP showed a slight increase with age with, for instance, the mean values found for men and women of 41–51 years being 60% and 50% higher, respectively, than in men and women of 20–30 years (Table 1); these differences are significantly different ($P<0·05$). The increase of serum FABP with age most likely relates to the fact that FABP is eliminated from the circulation predominantly by renal clearance[3,5] and that renal function generally decreases with age.[13] In addition, release of FABP from muscle tissue may increase with age. For plasma myoglobin, which is also cleared from the circulation mostly by the kidneys,[14] a similar increase with age has been found.[15,16]

For all subjects investigated the mean (SD) value of serum FABP was 1·6 (0·8) µg/L ($n=79$). This value agrees with studies of Tanaka *et al.*[3] who reported FABP in plasma from healthy subjects to vary between 0 and 2·8 µg/L ($n=86$), although recently the same investigators reported a higher reference concentration [3·6 (1·8) µg/L, mean (SD), $n=100$].[9] Our present value, however, is lower than that determined previously in our laboratory [9 (5 µg/L), $n=72$].[5] The latter difference may relate to the use in the ELISA at that time of less specific polyclonal antibodies when compared to the present monoclonal antibodies, which would lead to overestimated values in case of low predilution of samples (fivefold predilution was used). The lower sensitivity of the previous assay may also have contributed to this difference.

Evaluation

A non-competitive sandwich ELISA for FABP was developed which is based on the use of monoclonal capture and detector antibodies recognizing different epitope groups on the protein molecule. This permits the simultaneous incubation of the FABP-containing serum or plasma sample with the (immobilized) capture antibody and the (conjugated) detector antibody, thus limiting the number of steps of the assay procedure. In addition, an incubation time of 30 min appeared sufficient, which relates to

the antibodies having been selected for a high affinity for FABP. Since detection of the HRP conjugate is very rapid (<7 min), the entire procedure, including washing steps and reading of the absorbance, can be performed routinely within 45 min, which is markedly faster than the 5 h needed to complete the previously developed polyclonal antibody-based immunochemical assay.[5]

The newly developed immunoassay showed a low intra-assay and inter-assay variation and an excellent comparability with the previously described assay for FABP.[5] The detection limit (0·2 μg/L) is markedly lower than that of reported direct[5,9,17] or competitive immunoassays,[2,3] and is sufficiently low to permit the accurate determination of FABP concentrations in plasma from healthy individuals. The measuring range allows samples from patients with AMI to be routinely diluted 10-fold (20-fold final dilution in the assay) and include >90% of reported FABP concentrations in pathological samples (4–120 μg/L).[2,3,5–8] The use of monoclonal antibodies ensures a source of antibodies of constant quality so that the assay can be easily automated. In view of the good performance of FABP as plasma marker in the early phase diagnosis of acute myocardial infarction, the immunoassay described here may well be used for determination of FABP in blood from patients entering the coronary care emergency room of the hospital, and will also be useful as reference assay for even more rapid assay systems such as an FABP immunosensor currently being developed.[18]

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Urinary fatty acid–binding protein as a new clinical marker of the progression of chronic renal disease

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Previous studies have indicated that in massive proteinuria, free fatty acids (FFAs) bound to albumin were overloaded in the proximal tubule and exacerbated tubulointerstitial damage. Liver-type fatty acid–binding protein (L-FABP) is an intracellular carrier protein of FFAs that is expressed in the proximal tubule of human kidney. We sought to evaluate urinary L-FABP as a clinical marker in chronic renal disease. Urinary L-FABP was measured in patients with nondiabetic chronic renal disease (n = 120) with the use of a newly established ELISA method. We then monitored these patients for 15 to 51 months. Clinical data were analyzed with multivariate analysis. Urinary L-FABP was correlated with urinary protein, urinary α_1-microglobulin, and serum creatinine concentrations. Urinary L-FABP at the start of follow-up (F = 17.1, r = .36, P < .0001) was selected as a significant clinical factor correlated with the progression rate, defined as a slope of a reciprocal of serum creatinine over time. We next selected the patients with mild renal dysfunction (n = 35) from all 120 patients and divided them into 2 groups according to progression rate: the progression group (n = 22) and the nonprogression group (n = 13). Serum creatinine and urinary protein concentrations and blood pressure at the start of follow-up were higher in the progression group than in the nonprogression group, although we detected no significant difference between the 2 groups. Urinary L-FABP was significantly higher in the former group than in the latter (P < .05). The results showed that urinary L-FABP reflected the clinical prognosis of chronic renal disease. Urinary L-FABP may be a clinical marker that can help predict the progression of chronic glomerular disease. (J Lab Clin Med 2004;143:23-30)

Abbreviations: BSA = bovine serum albumin; ELISA = enzyme-linked immunosorbent assay; FFA = free fatty acid; L-FABP = liver-type fatty acid–binding protein; mAb = monoclonal antibody; α_1-MG = α_1-microglobulin; NAG = *N*-acetyl-β-D-glucosaminidase; PBS = phosphate-buffered saline solution

Increasing clinical and experimental evidence shows that the progressive nature of chronic glomerular disease depends significantly on tubulointerstitial involvement.[1] Recent studies have shown that urinary protein has renal toxicity and contributes to the progression of renal damage by causing tubulointerstitial disease.[2-6] FFAs bound to albumin[7,8] may play a role in the generation of tubulointerstitial disease. In massive proteinuria, FFAs are overloaded in the proximal tubule and induce inflammatory factors such as macrophage chemotactic factors,[9] which in turn aggravate urinary protein–related tubulointerstitial damage.[10-12] We showed that FFAs may be responsible for 1 mechanism leading to tubulointerstitial damage seen in massive proteinuria.[13] FFAs are overloaded in the proximal tubule not only in massive proteinuria but also in various other kinds of stresses to the proximal tubule such as ischemia[14] and toxic insults,[15] both of which have been implicated in the progression of renal disease.

FFAs loaded in the proximal tubule are believed to

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Table I. Clinical and laboratory findings

Variable	$n = 120$
Age (yr)*	45 ± 15
Sex (%)	
Male	77 (64)
Female	43 (36)
Mean blood pressure (mm Hg)*	96 ± 14
Serum creatinine (mg/dL)*	1.0 ± 0.4
Total cholesterol (mg/dL)*	202 ± 41
Triglyceride (mg/dL)*	143 ± 93
Urinary protein (g/g · cr)*	1.1 ± 1.3
Urinary NAG (IU/g · cr)*	7.6 ± 6.1
Urinary α1-MG (mg/g · cr)*	9.7 ± 9.9
Urinary L-FABP (μg/g · cr)*	30.1 ± 38.9
Renal biopsy (%)	89 (74)
Minor glomerular abnormalities	15
Mesangial proliferative glomerulonephritis	49
Membranous nephropathy	6
Focal segmental glomerulosclerosis	18
Membranoproliferative glomerulonephritis	1
Medication†	
On	73
Off	47

*Data expressed as mean ± SD.
†Medication included an angiotensin-converting enzyme inhibitor, an angiotensin-1 receptor antagonist, and steroids.

be bound to cytoplasmic fatty acid–binding protein and transported to mitochondria or peroxisomes, where they are metabolized by β-oxidation. In the human proximal tubules, L-FABP of 14.4 kD is expressed.[16] Because renal L-FABP has not yet been investigated in patients with renal disease, we sought to clarify the clinical relevance of urinary excretion of L-FABP in chronic glomerular disease after developing specific mAbs against human L-FABP.

METHODS

Patient selection. We selected 120 nondiabetic adult patients from the outpatient clinic of the University of Tokyo Hospital between 1997 and 2000 on the basis of a serum creatinine concentration less than 2.5mg/dL. Relevant clinical parameters were monitored every month or second month for more than 15 months, with no change in medication. In each patient the diagnosis of chronic glomerular disease had been made on the basis of renal biopsy ($n = 89$) or clinical history, and none had clinical or laboratory evidence of underlying systemic disease. Table I summarizes the clinical and laboratory findings of the subjects. This research was carried out in accordance with the principles of the Declaration of Helsinki; informed consent was obtained and our institutional review board approved the study.

Development of specific mAbs against human L-FABP. BALB/C mice were subcutaneously injected once with 50 μg of purified recombinant human L-FABP in Freund's complete adjuvant, and the same dose was injected after 2 weeks. We prepared recombinant human L-FABP using the fusion plasmid system (pMAL-cRI).[17] Spleen cells from immunized mice were fused with the murine myeloma line P3/X63-AG8 6.5.3.[18] We selected hybridomas in a hypoxanthine aminopterin thymidine medium and screened for antibody production using an ELISA and the purified recombinant L-FABP–coated plate. We obtained 18 positive clones by limiting dilution. Expanded cultures from 2 hybridomas, FABP-2 and FABP-L, were injected into the peritoneal cavities of pristane-primed mice, after which we collected ascitic fluid and fractionated it into IgG by means of protein A column chromatography. mAb FABP-2 was conjugated to horseradish peroxidase with the use of succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate, in accordance with the instructions of the manufacturer (Pierce Chemical Co, Rockford, IL).

Establishment of ELISA for urinary L-FABP. We coated 96-well microtiter plates with 10 mg/L mAb FABP-L and incubated them overnight. Unreacted sites were blocked with PBS containing 10 g/L BSA overnight. The plates were then washed 3 times with PBS containing 0.5 g/L Tween-20 with 1 g/L BSA and dried.

We incubated 100 μL of properly diluted standards or samples in the wells of each plate at room temperature for 1 hour. They were then washed 4 times with PBS containing 0.5 g/L Tween-20 and allowed to react with 100 μL of horseradish peroxidase–conjugated FABP-2 for 1 hour. After 4 more washes, 100 μL of enzyme substrate (*O*-phenylenediamine/H_2O_2) solution was reacted at room temperature for 30 minutes, after which the reaction was terminated with the addition of 100 μL of 2 mol/L sulfuric acid. Absorbance was measured at 492 nm on a microplate reader.

We prepared standards for the assay by measuring the protein concentration of purified recombinant L-FABP using Lowry's method and adjusting to make up a series ranging from 0 to 400 ng/mL with PBS containing 10 g/L BSA.

The reference value of urinary excretion of L-FABP was set after analysis of urine samples from healthy volunteers at Eiken Chemical Co Ltd ($n = 97$). None of the volunteers had a history of renal disease or abnormal finding on urinalysis.

Clinical parameters of serum and ambulatory spot urine samples. Creatinine, total cholesterol, and triglyceride concentrations were measured in serum; L-FABP, creatinine, total protein, NAG, and α_1-MG concentrations were measured in urine. We used enzymatic methods to measure serum and urinary creatinine, serum cholesterol, and triglyceride; the pyrogallol red–molybdate complex method to measure urinary protein; chlorophenol red–NAG as substrate to measure urinary NAG; and the latex aggregation assay to measure α_1-MG. Serum creatinine was measured every month or second month in the study patients.

We divided the patients into 3 groups on the basis of urinary protein level: The "mild proteinuria" group ($n = 76$) consisted of patients with urinary protein of less than under 1 g/g · cr, the "moderate proteinuria" group ($n = 33$) comprised patients with a value of 1 to 3 g/g · cr, and the "heavy proteinuria" group ($n = 11$) consisted of patients with values of more than 3 g/g · cr. We compared 3 groups with the level of urinary L-FABP.

Clinical course and urinary L-FABP. In an effort to evaluate the progression of renal disease, we analyzed the relationship between time (months) and the reciprocal of serum creatinine.[19-26] The progression rate of renal disease was defined as a slope of the regression line. In the patients whose serum creatinine fluctuated within the normal range during the follow-up period, the progression rate was evaluated as zero because the slope of the regression line did not represent the progression of renal disease in those patients.

Moreover, we selected the patients with mild renal dysfunction whose serum creatinine values were greater than the normal range (1.2 mg/dL in men, 0.9 mg/dL in women) from all the patients shown in Table I (n = 35). We divided these patients into 2 groups: The first group consisted of patients showing a slope of the regression line of less than −.001 (dL · mg^{-1} · $month^{-1}$) (n = 22), the average of all 120 patients, and the second group comprised patients showing a slope of the regression line of more than −.001 (dL · mg^{-1} · $month^{-1}$) (n = 13). We compared the 2 groups with regard to clinical parameters at the start of follow-up, histologic diagnosis, presence or absence of medication, and duration of follow-up.

Statistical analysis. Data are expressed as the mean ± SD. To clarify the relationship between urinary L-FABP and clinical parameters and the relationship between the progression rate and clinical parameters, we applied multiple-regression analysis based on the stepwise method. Differences in parameters among the 3 groups were analyzed with Scheffé's multiple-comparison procedure after the Kruskal-Wallis test was performed. To compare the parameters from the 2 groups, we used the Mann-Whitney U test for unpaired data and the Wilcoxon rank-sum test for paired data. We conducted comparisons of categorical variables between the 2 groups using Fisher's exact probability test. These statistical analyses were performed with a software program for the Macintosh operating system (Stat View 5.0; SAS Institute, Inc., Cary, NC). P values of less than .05 were considered statistically significant.

RESULTS

ELISA for urinary L-FABP. The within-assay variance was between 6.1%, 4.9%, and 3.8% (coefficient of variation) when 8 replicates were performed on urine samples with L-FABP concentrations of 13.5, 45, and 125 ng/mL, respectively. We evaluated between-assay variability by measuring the same 3 samples in each plate for 10 days. The coefficients of variation were 6.6%, 4.2%, and 5.1%, respectively. The recovery of L-FABP values was between 90% and 100% when purified recombinant L-FABP was added in concentrations of 50, 100, and 200 ng/mL to urine samples. Serial dilutions of urine samples with L-FABP concentrations of 15, 94, and 270 ng/mL were linear.

The reference value of urinary excretion of L-FABP was analyzed in 26 male and 71 female volunteers. The average age of our healthy volunteers was 33.0 ± 7.5 years, and no sex-related statistical difference was detected. The reference value of urinary L-FABP was 6.5 ± 5.4 ng/mL, or 5.2 ± 3.6 μg/g · cr in healthy volunteers (n = 97), whereas the level of urinary L-FABP was 31.9 ± 48.0 ng/mL, or 30.1 ± 38.9 μg/g · cr in the patients with chronic glomerular disease (n = 120). The urinary L-FABP concentration was significantly higher in the patients with chronic glomerular disease than in healthy volunteers (P < .0001).

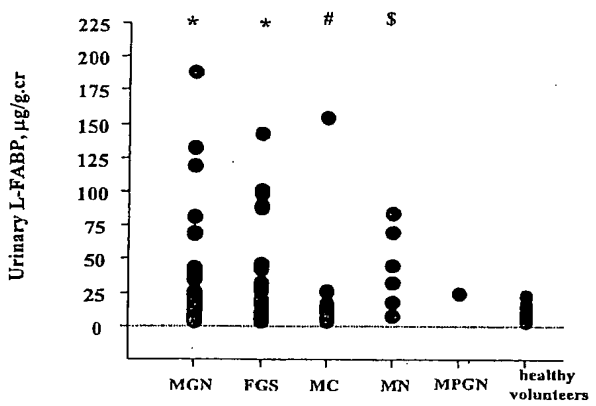

Fig 1. Distribution of urinary excretion of L-FABP in healthy volunteers and patients with various renal diseases. *MGN* = patients with mesangial proliferative glomerulonephritis; *FGS* = patients with focal segmental glomerulosclerosis; *MC* = patients with minor glomerular abnormalities; *MN* = patients with membranous nephropathy; *MPGN* = patient with membranoproliferative glomerulonephritis. *P < .0001 vs healthy volunteers; $$P$ < .0005 vs healthy volunteers; #P < .05 vs healthy volunteers.

We selected the patients with chronic glomerular disease diagnosed on the basis of renal-biopsy findings (n = 89) (Fig 1). Compared with urinary excretion of L-FABP in healthy volunteers, urinary excretion of L-FABP was significantly increased in the patients with mesangial proliferative glomerulonephritis (25.5 ± 36.7 μg/g · cr; P < .0001, n = 49), focal segmental glomerulosclerosis (42.9 ± 42.7 μg/g · cr; P < .0001, n = 18), membranous nephropathy (43.0 ± 29.6 μg/g · cr; P < .0005, n = 6), and minor glomerular abnormalities (18.8 ± 37.4 μg/g · cr; P < .05, n = 15). The urinary L-FABP concentration in the patients with membranoproliferative glomerulonephritis was 24.6 μg/g · cr (n = 1).

Clinical parameters correlated with urinary L-FABP. When we conducted a stepwise regression analysis using urinary L-FABP as a dependent variable and 6 clinical independent variables (serum creatinine, serum total cholesterol, serum triglyceride, urinary protein, urinary NAG, and urinary α_1-MG), urinary L-FABP was found to be significantly correlated with urinary protein (F = 24.2), urinary α_1-MG (F = 13.1), and serum creatinine (F = 11.9) (r = .76, P < .0001; Table II).

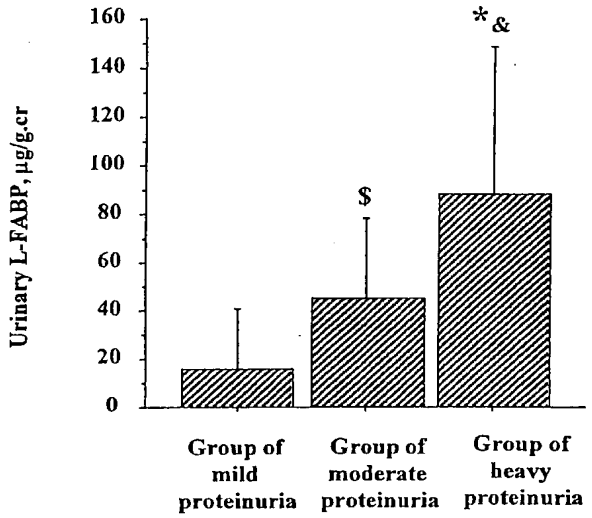

Fig 2. Urinary excretion of L-FABP in the patients divided into 3 groups on the basis of urinary protein concentration. Urinary excretion of L-FABP was significantly greater in the heavy-proteinuria group than in the mild- and moderate-proteinuria groups. Urinary excretion of L-FABP was significantly greater higher in the moderate-proteinuria group than in the mild-proteinuria group.
*$P < .0001$ vs mild-proteinuria group; $\$P < .0005$ vs the mild-proteinuria group; $\&P < .001$ vs the moderate-proteinuria group.

Table II. Stepwise regression analysis for urinary excretion of L-FABP

Independent variables	F ratio
Serum creatinine	11.9*
Total cholesterol	0.01
Triglyceride	0.23
Urinary protein	24.2*
NAG	0.03
α_1-MG	13.1*

*Statistically significant.

In the heavy-, moderate-, and mild-proteinuria groups, urinary L-FABP levels were 88.2 ± 60.4, 43.9 ± 34.4, and 15.8 ± 25.0 μg/g · cr, respectively. Urinary L-FABP levels in the heavy-proteinuria group were significantly higher than those in the moderate-proteinuria group ($p < .001$) or those in the mild-proteinuria group ($P < .0001$). Urinary L-FABP levels in the moderate-proteinuria group were significantly higher than those in the mild-proteinuria group ($P < .0005$; Fig 2).

Clinical course and urinary L-FABP. Patients were monitored for 27.3 ± 9.4 months (range 15–51 months). The slope-of-regression line was −.001 ± .004 (dl · mg^{-1} · $month^{-1}$). In the stepwise regression analysis with 10 clinical independent variables (urinary excretion of L-FABP, serum creatinine, serum total cholesterol, serum triglyceride, urinary protein excretion, urinary NAG, urinary α_1-MG, age, mean blood pressure, duration of follow-up) on all of the patients shown in Table 1, the progression rate was correlated only with the urinary excretion of L-FABP ($F = 17.1$, $r = .36$, $P < .0001$; Table III). Neither sex nor the presence of medication influenced the progression rate (data not shown). We selected the patients with mild renal dysfunction described above from the patients shown in Table 1 and divided them into 2 groups, the first with progression of renal disease and the second group without progression (Table IV). We confirmed that the slope-of-regression line was significantly lower in group 1 than in group 2 ($P < .0001$; Fig 3, *A* and *B*). In the first group, serum creatinine was significantly increased, from 1.7 ± .4 to 2.5 ± 1.5 mg/dL, at the end of follow-up, 26.1 ± 8.6 months ($P < .0001$; Fig 3, *C*).

Table III. Stepwise regression analysis for progression rate of renal disease

Variables	F ratio
Serum creatinine	1.5
Total cholesterol	1.5
Triglyceride	0.2
Urinary protein	2.0
NAG	0.1
α1-MG	0.2
L-FABP	17.1*
Age	0.1
Mean blood pressure	0.4
Duration of follow-up	0.0

*Statistically significant.

Table IV. Comparison of background in group 1 (progression group) and group 2 (nonprogression group)

Variable	Group 1 ($n = 22$)	Group 2 ($n = 13$)
Age (yr)*	51 ± 17	50 ± 10
Sex		
Male	17	8
Female	5	5
Follow-up (mo)*	26.1 ± 8.6	23.8 ± 12.5
Histologic diagnosis		
Minor glomerular abnormalities	2	0
Mesangial proliferative glomerulonephritis	5	7
Membranous nephropathy	1	1
Focal segmental glomerulosclerosis	6	0
Unknown	8	5

*Data expressed as mean ± SD.

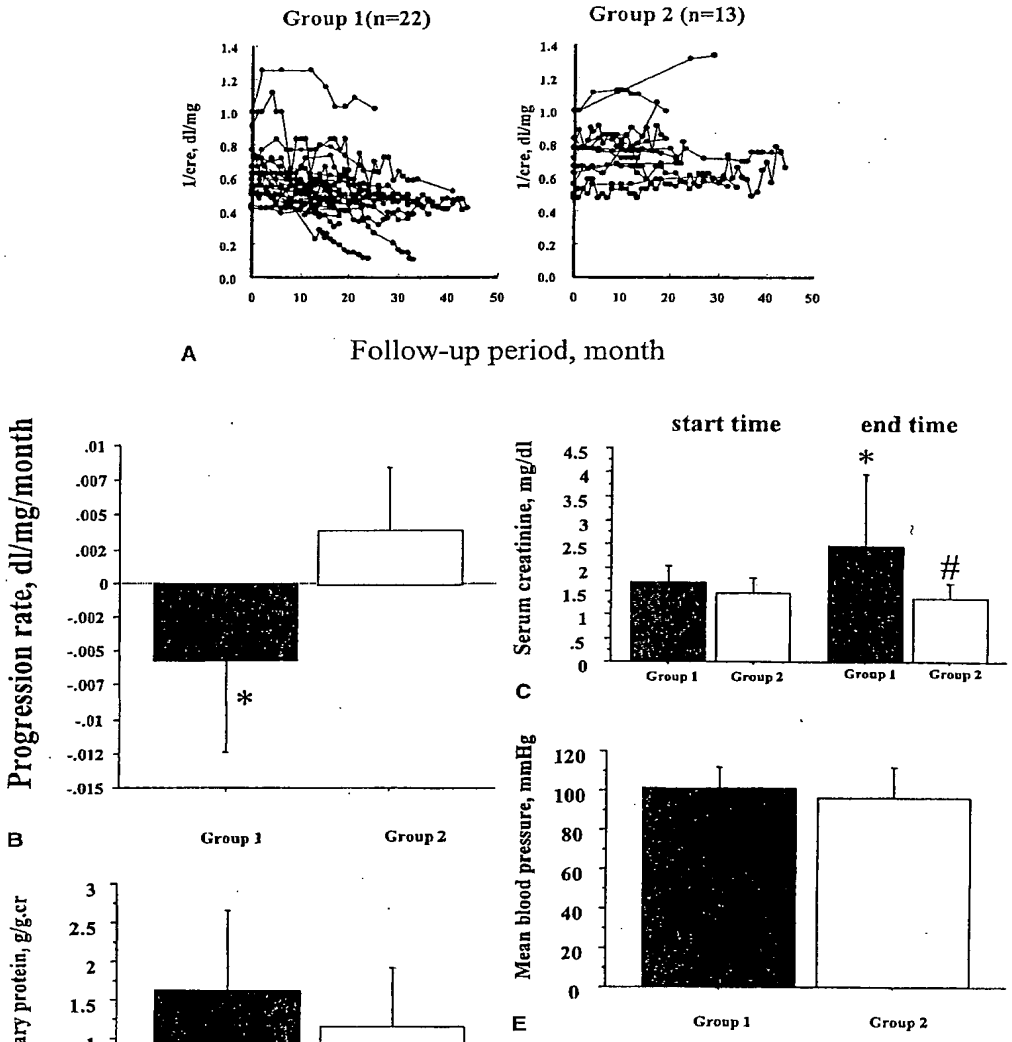

Fig 3. Comparison of the progression rate and clinical parameters in groups 1 (disease progression) and 2 (nonprogression). A, Reciprocal of serum creatinine. The *vertical axis* shows the reciprocal of serum creatinine, and the *horizontal axis* shows the follow-up periods for groups 1 and 2. B, Progression rate in groups 1 and 2. Progression rate was confirmed to be significantly slower in group 1 than in group 2. $*P < .0001$ vs group 2. C, Serum creatinine levels in groups 1 and 2 at the start and end of follow-up. Serum creatinine at the start of follow-up was not significantly different between the groups. In group 1, serum creatinine concentration was significantly increased, from 1.7 ± 0.4 to 2.5 ± 1.5 mg/dL, at the end of follow-up, 26.1 ± 8.6 months. In group 2, serum creatinine was significantly decreased, from 1.4 ± 0.4 to 1.3 ± 0.3 mg/dL, at the end of follow-up, 23.8 ± 12.5months. $*P < .0001$ vs group 1 at the start of follow-up; $\#P < .05$ vs group 2 at the start of follow-up. D, Urinary protein levels in groups 1 and 2. Urinary protein at the start of follow-up was not significantly different between groups 1 and 2. E, Mean blood pressure in groups 1 and 2. Blood pressure at the start of follow-up was not significantly different between groups 1 and 2. F, Urinary L-FABP levels in groups and 2. Urinary L-FABP was significantly higher in group 1 than in group 2. $\#P < .05$ vs group 2.

Meanwhile, in the second group, serum creatinine was significantly decreased, from 1.4 ± .4 to 1.3 ± .3 mg/dL, at the end of the follow-up, 23.8 ± 12.5 months ($P < .05$; Fig 3, *C*). Serum creatinine (Fig 3, *C*), urinary protein (Fig 3, *D*), and mean blood pressure (Fig 3, *E*) at the start of follow-up were higher in group 1 than in group 2, although the difference was not significant. Only urinary L-FABP was significantly higher in the former group than in the latter ($P < .05$; Fig 3, F). Sex, histologic diagnosis, the presence or absence of medication, and duration of follow-up were not significantly different between the 2 groups.

DISCUSSION

We developed specific mAbs against human L-FABP to clarify the clinical relevance of urinary L-FABP in chronic glomerular disease. We have shown urinary L-FABP to be a clinical parameter that reflects the progression of renal disease and have determined that this value was higher in the group demonstrating progression of renal disease than in the patients in whom disease was not progressing. Therefore we propose that urinary L-FABP has promise as a clinical marker to predict and monitor the progression of chronic glomerular disease. Such a predictor will contribute to the treatment of renal disease.

To investigate the clinical significance of urinary L-FABP, we measured urinary L-FABP using ambulatory spot urine samples because these samples were easy to obtain in the outpatient clinic and contamination of such samples is less than that in 24-hour urine collections. The Cockroft-Gault formula, or MDRD formula, for the evaluation of creatinine clearance is not suitable in Japanese patients, whose urinary excretion of creatinine decreases less with age than it does in Europeans and Americans.[27] We therefore defined progression of renal disease as the slope of the reciprocal of serum creatinine.[23-26]

In the human kidney, 2 types of FABP have been localized.[16] One, the liver type, is expressed in the proximal tubule; the other, a heart type, is expressed in the distal tubule. L-FABP, a small protein of 14.4 kD, is a carrier protein that transports fatty acids to mitochondria or peroxisomes, where fatty acids are metabolized by way of β-oxidation. The transcription of L-FABP gene is promoted by FFAs.[28] Recent evidence suggests that L-FABP transports FFAs from the cytosol to the nucleus[29,30] and interacts with the nuclear protein, peroxisome proliferator–activated receptor,[31] which is a nuclear target for FFAs and initiates gene expression of enzymes involved in lipid metabolism.[32,33] L-FABP may thus play a key role in fatty-acid metabolism in the proximal tubules, being induced by fatty acids per se.

As FFAs are bound to serum albumin,[7,8] filtered through glomeruli, and reabsorbed into the proximal tubule along with albumin, fatty acids are overloaded in the proximal tubule in massive proteinuria. Nonoxidized fatty acids appear to be cytotoxic by peroxidation[34,35] and induce some chemoattractants from the proximal tubules,[9] which in turn progress to tubulointerstitial damage.[13] These are supported by clinical findings that proteinuria is a major factor in the progression of renal disease.[2-6]

Tubulointerstitial inflammation induced by lipid toxicity may be provoked not only by proteinuria but also by other stresses such as ischemia[34,35] and toxins.[36,37] We therefore hypothesize that various stresses to the proximal tubule tend to overload fatty acids in the cytoplasm and thereby damage tubules with the release of inflammatory factors. In this way, tubulointerstitial inflammation is provoked and renal function deteriorates over time.

Because we detected a significant correlation of urinary L-FABP with urinary protein, we assumed that urinary protein induced excretion of L-FABP. To confirm this supposition, we prepared human L-FABP gene transgenic mice and made a protein-overload model. In control transgenic mice, human L-FABP was immunohistochemically identified in the proximal tubule and not detectable in urine. In the protein-overload model, Northern-blot analysis showed up-regulation of human L-FABP gene expression and massive amounts of L-FABP were excreted into urine (unpublished observations). The results suggest that stresses on the proximal tubules, such as urinary protein, induce up-regulation of human L-FABP gene expression and accelerate excretion of L-FABP from the proximal tubule, resulting in an increase in urinary excretion of L-FABP. From the clinical findings reported herein and the experiments involving transgenic mice, we hypothesize that a variety of stresses, such as massive proteinuria and ischemia, cause an overload of FFAs in the proximal tubules and exacerbate tubulointerstitial damage. L-FABP gene expression is increased and L-FABP accelerates fatty-acid metabolism by carrying them to mitochondria or peroxisomes. Moreover, the results suggest that L-FABP helps maintain a low level of fatty acids in the cytoplasm by being excreted from the proximal tubules into urine together with fatty acids. This mechanism should be clarified in a future study.

It is widely confirmed that quantification of urinary protein helps physicians predict the risk of disease progression and the risk of dialysis for individual patients.[5,6] However our results show that the progression rate was less correlated with urinary protein than with urinary excretion of L-FABP. Our patients' average urinary protein value was relatively modest and their

characterization might be limited. To demonstrate the exact diagnostic validity of urinary L-FABP as a predictor, we are planning a multiple-institution study.

In conclusion, urinary excretion of L-FABP may reflect various kinds of stresses that cause tubulointerstitial damage, such as those caused by urinary protein on the proximal tubule, and may therefore be unique clinical marker of the progression of chronic renal disease.

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